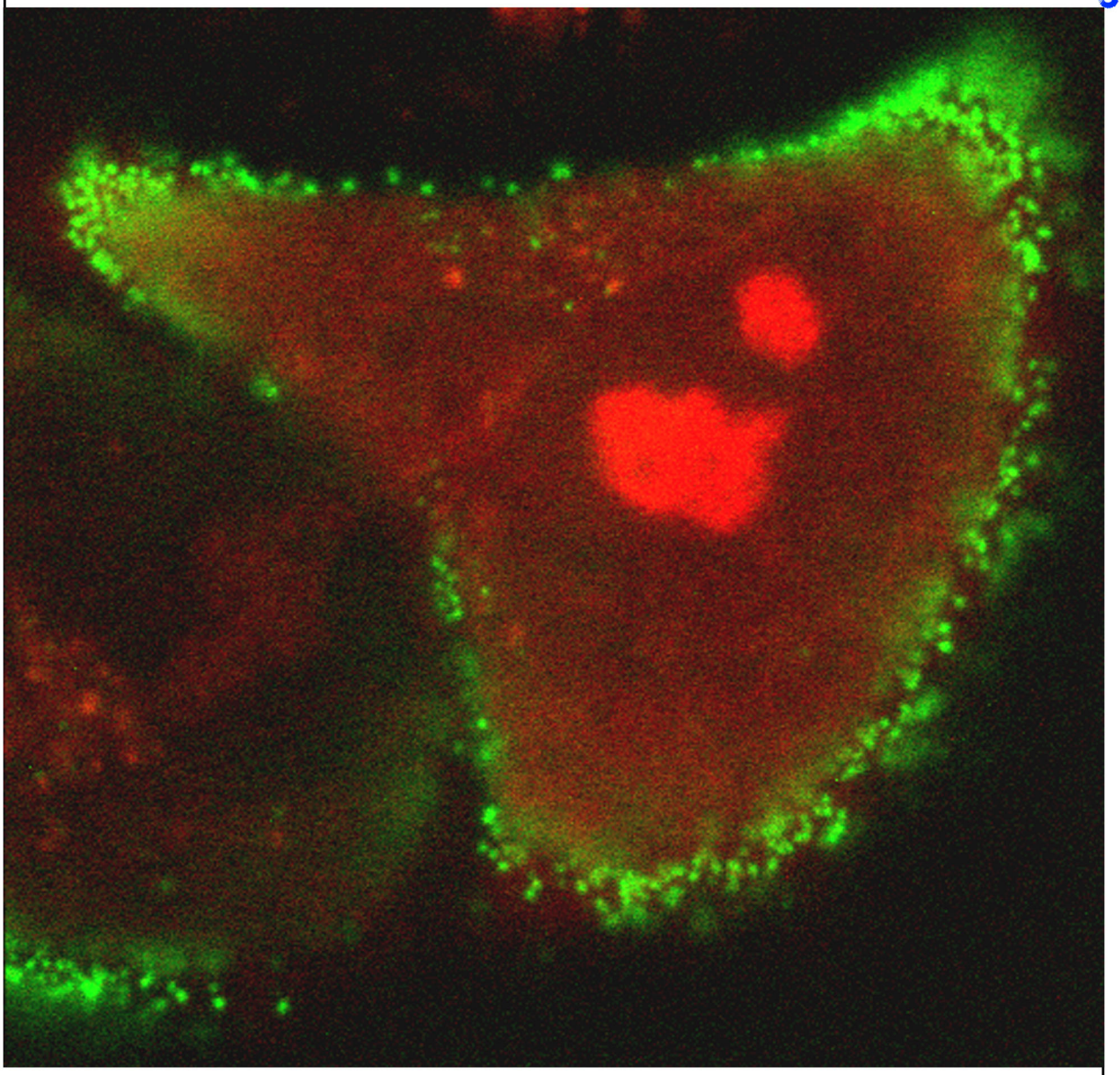
Targets and Mechanisms

7th Annual Symposium on Antiviral Drug Resistance



Program and Abstracts

November 12-15, 2006

Westfields Conference Center
Chantilly, Virginia

Sponsored by the University of Pittsburgh
Co-sponsored by the HIV Drug Resistance Program, NCI

On the cover:

Fluorescence microscopic image demonstrating HIV-2 Gag–Gag interactions via bimolecular fluorescence complementation in HeLa cells. The *gag* gene was fused to either the N-terminal portion or the C-terminal portion of the yellow fluorescence protein (YFP) gene. Gag–Gag interactions brought the two halves of YFP into close proximity allowing bimolecular complementation and YFP fluorescence (shown in green). The red fluorescence is from Rev fused to mCherry fluorescence protein. This figure was provided by Vitaly Boyko and Wei-Shau Hu (HIV Drug Resistance Program, National Cancer Institute); similar images and interactions were also seen with HIV-1 and HIV-2 Gag proteins, as described in the abstract on page 35.

Targets and Mechanisms

7th Annual Symposium on Antiviral Drug Resistance

Sunday, November 12 – Wednesday, November 15, 2006

Symposium Organizers

John W. Mellors, M.D.
Division of Infectious Diseases
University of Pittsburgh

John M. Coffin, Ph.D.
Tufts University
(on contract to NCI)

Stephen H. Hughes, Ph.D.
HIV Drug Resistance Program
National Cancer Institute

Sunday	6:00 pm	Session 1: Introduction and Overviews
	7:35 pm	Reception and Dinner
Monday	9:00 am	Session 2: Viral Entry
	12:00 pm	Lunch
	1:00 pm	Session 3: Biology of Viral Infection
	2:30 pm	Session 4: Assembly, Release, and Processing
	6:00 pm	Poster Session and Reception
	7:30 pm	Dinner
Tuesday	9:00 am	Session 5: Novel Antiviral Strategies
	12:00 pm	Lunch
	1:00 pm	Session 5 (cont'd)
	5:00 pm	Poster Session and Reception
	6:30 pm	Dinner
Wednesday	9:00 am	Session 6: Nucleic Acid Replication
	12:10 pm	Lunch and Adjournment

Oral sessions are located in the *Lincoln Forum Amphitheater*

Poster sessions are located in the *Washingtonian Room*

All meals are in the *Fairfax Room;* on Monday through Wednesday, breakfast is served from 7:00 to 8:30 am

Please note: Use of recording devices (including cameras, video, and tape recorders) by participants is not permitted during the oral and poster sessions.

Program at a Glance

FOREWORD

Effective antiviral therapy is the only hope for survival or alleviation of disease for millions of Americans and tens of millions of individuals worldwide suffering from chronic viral infection, including those caused by HIV, HBV, HCV, and others. In addition, the threat of global outbreaks continues from influenza, SARS, and other respiratory viruses. Despite considerable progress in the development of effective inhibitors directed at specific aspects of viral life cycles, therapeutic efficacy has been limited by the evolution of resistant virus. This problem not only results in the failure of therapy, but may limit the effectiveness of subsequent therapies. Moreover, attempts to counter drug resistance lead to complex, expensive, and toxic regimens. Antiviral drug resistance is therefore of paramount importance in dealing with growing epidemics of virus infection.

The Symposium on Antiviral Drug Resistance: Targets and Mechanisms brings together researchers in a variety of virus systems to exchange new information on viral targets for therapy, on antiviral drugs, and on resistance to these drugs. We believe that understanding the similarities and differences of the diverse viral systems will lead to new insights into the problem of resistance in each individual virus.

The Symposium is sponsored by the University of Pittsburgh and co-sponsored by the HIV Drug Resistance Program (HIV DRP). The HIV DRP was established in 1997 as part of the ongoing HIV/AIDS research activity in the National Cancer Institute. Centered at the NCI-Frederick campus, the Program also includes investigators in the NIH Clinical Center and collaborators in a number of academic centers, including the University of Pittsburgh.

ACKNOWLEDGEMENTS

We thank those who helped to make the 7th Annual Symposium on Antiviral Drug Resistance: Targets and Mechanisms possible:

- The University of Pittsburgh for providing the primary financial support
- The Center for Cancer Research, National Cancer Institute, for co-sponsorship
- Members of the HIV Drug Resistance Program for assistance and advice in many areas, in particular Anne Arthur and Susan Jordan
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7TH ANNUAL SYMPOSIUM ON ANTIVIRAL DRUG RESISTANCE: TARGETS AND MECHANISMS

Program[a]

SUNDAY, NOVEMBER 12, 6:00 – 7:35 PM

Session 1 Introduction and Overviews

Chair: **John Coffin**, Tufts University, Boston, MA (Special Advisor to the Director, Center for Cancer Research, National Cancer Institute)

J.N. Strathern, Center for Cancer Research, National Cancer Institute, NCI-Frederick, Frederick, MD: *Welcoming Remarks*

* B.H. Hahn, University of Alabama at Birmingham, Birmingham, AL: *Origins of HIV: Expect the Unexpected* Page 1

* D. Averitt Bridge, The Well Project, Atlanta, GA: *Impact of the HIV/AIDS Epidemic on Women: Personal Reflections*

Welcome Reception and Dinner

MONDAY, NOVEMBER 13, 9:00 AM – 12:00 NOON

Session 2 Viral Entry

Chair: **Hans-Georg Kräusslich,** University of Heidelberg, Heidelberg, Germany

* B. Dey, M. Tang, S. Phogat, Y. Li, A. Phogat, J. Guenaga, M. Forsell, and R. Wyatt, 2
Vaccine Research Center, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD: *Analysis of Antibody Responses to the HIV-1 Envelope Glycoproteins*

S. Subramaniam, R. Sougrat, A.E. Bennett, L. Hartnell, A. Bartesaghi, and J. Liu, 3
Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD: *Electron Tomography of Immunodeficiency Viruses and Mechanisms of Cellular Entry*

* P. Pugach, T.J. Ketas, S.E. Kuhmann, and J.P. Moore, Weill Medical College of Cornell 4
University, Department of Microbiology and Immunology, New York, NY: *The Mechanism of HIV-1 Resistance to Small Molecule CCR5 Inhibitors*

Coffee Break

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[a] Keynote talks are designated with an asterisk; presenters' names are underlined.

MONDAY, NOVEMBER 13, 1:00 – 2:30 PM

Session 3 Biology of Viral Infection

Chair: **Jeffrey Lifson,** AIDS Vaccine Program, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD

MONDAY, NOVEMBER 13, 2:30 – 5:40 PM

Session 4 Assembly, Release, and Processing

Chair: **Irene Weber,** Georgia State University, Atlanta, GA

MONDAY, NOVEMBER 13, 6:00 – 7:30 PM

POSTER SESSION AND RECEPTION

Posters in this session will also be displayed on November 14 (see abstracts on pages 43-90).

TUESDAY, NOVEMBER 14, 9:00 AM – 4:40 PM

Session 5 Novel Antiviral Strategies

Co-Chairs: **Judith G. Levin,** National Institute of Child Health and Human Development, NIH, Bethesda, MD
Henry Levin, National Institute of Child Health and Human Development, NIH, Bethesda, MD

Coffee Break

TUESDAY, NOVEMBER 14, 5:00 – 6:30 PM

POSTER SESSION AND RECEPTION

Posters in this session will also be displayed on November 13 (see abstracts on pages 43-90).

WEDNESDAY, NOVEMBER 15, 9:00 AM – 12:10 PM

Session 6 Nucleic Acid Replication

Chair: **Daria Hazuda,** Merck Research Laboratories, West Point, PA

Coffee Break

POSTER PRESENTATIONS

AUTHOR INDEX [a]

[a] *Numbers in boldface indicate presenting authors.*

ORIGINS OF HIV: EXPECT THE UNEXPECTED

Beatrice H. Hahn

University of Alabama at Birmingham

Identification of the primate source that spawned the AIDS pandemic is of scientific and public health importance. To this end, we have recently used novel non-invasive methods to trace the origins of pandemic (group M) and non-pandemic (group N) HIV-1 to geographically isolated chimpanzee communities (*Pan troglodytes troglodytes*) in southern Cameroon. We have also identified a new ape reservoir that may have served as the source of HIV-1 group O. Our findings demonstrate how endangered primates can be studied by non-invasive molecular approaches to elucidate the epidemiological circumstances and mechanisms of pathogen transmission and adaptation.

ANALYSIS OF ANTIBODY RESPONSES TO THE HIV-1 ENVELOPE GLYCOPROTEINS

Barna Dey, Min Tang, Sanjay Phogat, Yuxing Li, Adhuna Phogat, Javier Guenaga, Mattias Forsell and Richard Wyatt

Vaccine Research Center, NIAID, NIH, Bethesda, MD 20892

HIV-1 evades host humoral responses to surface exposed envelope glycoproteins by glycan shielding, conformational masking, spike dissociation, immunodominant variable loops and occlusion of conserved regions of both the exterior gp120 envelope glycoprotein and the transmembrane gp41 glycoprotein. Because of these generally successful immune escape mechanisms, natural infection rarely elicits antibodies that can penetrate viral defenses and neutralize a broad array of primary isolates. Data will be presented of selected patient sera that demonstrate breadth of neutralization that we are analyzing to better understand the regions targeted on the Env spike. In parallel, using atomic-level structural information coupled to biochemical, biophysical, antigenic and immunogenic analysis, we have sought to create novel protein immunogens capable of eliciting a neutralizing antibody response against multiple HIV-1 primary isolates. Strategies include stabilizing gp120 in the CD4-bound conformation by the introduction of cavity-filling mutations and internal cysteine pairs and to mimic the highly conserved membrane proximal region of gp41 to which two broadly neutralizing antibodies bind (2F5 and 4E10) The coupling of the analysis of antibodies elicited during HIV-1 natural infection with atomic-level structural analysis culminating with immunogen characterization in vitro and in vivo provides a new paradigm for the generation of improved immunogens that more closely mimic relevant properties of the HIV-1 Env spike.

ELECTRON TOMOGRAPHY OF IMMUNODEFICIENCY VIRUSES AND MECHANISMS OF CELLULAR ENTRY

Sriram Subramaniam, Rachid Sougrat, Adam E. Bennett, Lisa Hartnell, Alberto Bartesaghi and Jun Liu.

Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892

Emerging methods in three-dimensional biological electron microscopy provide powerful tools and great promise to bridge a critical gap in imaging in the biomedical size spectrum. This gap comprises a size range of considerable interest in biology and medicine that includes cellular protein machines, giant protein and nucleic acid assemblies, viruses and small bacteria. These objects are generally too large and/or too heterogeneous to be investigated by high resolution X-ray and NMR methods; yet the level of detail afforded by conventional light and electron microscopy is often not adequate to describe their structures at resolutions high enough to be useful in understanding the chemical basis of biological function.

We are using a variety of approaches that utilize 3D electron microscopic imaging to analyze the structure of SIV and HIV in the purified state and at various stages of maturation in infected T-lymphocytes and macrophages. Thus, we are carrying out cryo electron tomographic studies of intact, unstained virions to understand the structure and distribution of envelope glycoproteins in SIV and HIV. We are also studying the 3D architectures of SIV and HIV virions captured at early and late stages of contact with CD4+ T-cells. In related studies, we are exploring the variation in conformation and stoichiometry of envelope glycoproteins in endosomal reservoirs of HIV-1 in infected macrophages. Current results from these structural analyses will be presented at the symposium.

Acknowledgements: We are grateful to Drs. Jeff Lifson, Eric Freed, and Robert Blumenthal at NCI, and Dr. Robert Buckheit at Imquest Inc. for their collaborations with us to enable our structural studies.

1. Subramaniam, S. (2005) Bridging the imaging gap: Visualizing subcellular architecture with electron tomography *Curr. Opin. Microbiology* 8*, 316-322.*
2. Subramaniam, S. (2006) The SIV surface spike imaged by electron tomography: One leg or three? *PLoS Pathog* 2, e91.

THE MECHANISM OF HIV-1 RESISTANCE TO SMALL MOLECULE CCR5 INHIBITORS

Pavel Pugach, Thomas J. Ketas, Shawn E. Kuhmann and John P. Moore

Weill Medical College of Cornell University, Department of Microbiology and Immunology, 1300 York Avenue, New York, NY 10021

Variants of HIV-1 that are resistant to small molecule CCR5 inhibitors have been generated *in vitro*. These resistant viruses continue to require CCR5 for replication in PBMC. We have previously examined their cross-resistance to other inhibitory CCR5 ligands and the genetics leading to resistance. In this study we sought to understand the mechanism by which these viruses continue to use CCR5 in the presence of high concentrations of a small molecule CCR5 inhibitor. We used flow cytometry to study the interactions between the HIV-1 inhibitory CCR5 ligands PSC-RANTES, PRO 140, PA12 and SCH-D. We then used clonal viruses bearing small molecule CCR5 inhibitor-sensitive and -resistant Env proteins in assays of viral replication in PBMC to determine how combinations of CCR5 ligands affected replication. SCH-D was found to inhibit PSC-RANTES induced CCR5 downregulation. PSC-RANTES alone efficiently inhibited replication of SCH-D-resistant viruses in PBMC. However, in the presence of SCH-D, the inhibitory action of PSC-RANTES on SCH-D-resistant viruses was significantly reduced. SCH-D was found to have little or no effect on the binding of the anti-CCR5 MAbs PRO 140 and PA12, and its ability to reverse their inhibition of the SCH-D-resistant viruses was correspondingly reduced. These results strongly suggest that the two SCH-D resistant viruses studied, although they follow different genetic pathways to resistance, can both use the CCR5-SCH-D complex for entry into target cells. Additional studies with Env-pseuodtyped viruses support this hypothesis and highlight differences in how resistance is manifested in PBMC and engineered cell lines. The latter results have implications for understanding commonly used, cell line-based assays for diagnosing resistance to CCR5 inhibitors.

EFFECTIVE SELECTION OF T-20 AND T-1249 RESISTANT HIV-1 VARIANTS BY A RANDOM MUTAGENESIS APPROACH

Raghavan Chinnadurai, Devi Rajan, Jan Münch, Frank Kirchhoff

Institute of Virology, University Clinic of Ulm, Albert-Einstein-Allee 11, D-89081, Ulm, Germany

Fusion inhibitors, such as T-20 (Enfuvirtide) and T-1249, block HIV-1 entry by preventing 6-helix-bundle formation of gp41 and represent a promising new class of antiretroviral drugs. It has been shown that mutations in the gp41 GIV motif mediate resistance against T-20. However, it has been proven difficult to generate viruses resistant against the 2nd generation fusion inhibitor T-1249, which is more potent than T-20 and active against most T-20 resistant viruses. To further assess HIV-1 resistance to fusion inhibitors and to analyze where changes in Heptad Repeat 1 might be tolerated we randomized 16 codons in the HIV-1 gp41 including those making contact with Heptad Repeat 2 and/or encoding residues in the GIV motif and the Hydrophobic Pocket. Infectivity assays demonstrated that only changes at positions 37I, 38V and 40Q near the N-terminus of HR-1 were well tolerated. Propagation of randomly mutagenized HIV-1 gp41 variants in the presence of high concentrations of T-1249 allowed the effective selection of highly resistant variants. Unexpectedly, all of them contained changes in the GIV motif. Interestingly, the extent of T-1249 resistance was inversely correlated to viral fitness. One HIV-1 mutant, however, showing ~10-fold reduced susceptibility to T-1249 inhibition replicated with wildtype-like kinetics and caused substantial CD4+ T cell depletion in ex vivo infected human lymphoid tissue. In summary, our results show that large parts of the HR-1 and HP in gp41 represent excellent targets for HIV-1 fusion inhibitors because changes are hardly tolerated and that our approach allows the effective selection of HIV-1 variants resistant against fusion inhibitors. Our data further demonstrate that the “GIV” motif also plays a key role in resistance to T-1249 and suggest that some HIV-1 variants resistant against 2nd generation fusion inhibitors might not be highly attenuated in vivo.

NEW TOOLS TO STUDY CELL-TO-CELL TRANSMISSION AND REPLICATION OF HUMAN T CELL LEUKEMIA VIRUS TYPE 1 (HTLV-1)

David Derse, Dmitriy Mazurov, Anna Ilinskaya, Patricia Lloyd, Shawn Hill, Gerald Princler, Michael Mitchell, and Gisela Heidecker

HIV Drug Resistance Program, Center for Cancer Research, NCI-Frederick

HTLV-1 is the causative agent of adult T cell leukemia (ATL) and an inflammatory disease of the central nervous system termed HAM/TSP. Of the approximately 20 million people infected with HTLV-1 worldwide, about 5% will develop either ATL or HAM/TSP. There is no viremia in HTLV-1-infected people, and virus is disseminated mainly by infected cells in the blood or breast milk. For technical reasons, quantitative studies of HTLV-1 replication in cell culture systems rely on cell-free virus infections. Although cell-to-cell transmission has been studied by microscopic image analysis for some viruses, parallel examination of virus replication has not been possible. In order to study virus replication in a system that requires coculture of virus producer cells with target cells, we have developed a new set of HTLV-1 vectors. These vectors are similar to those that were developed to study retrotransposition of mobile genetic elements in yeast and mammalian cells. The HTLV-1 transfer vector contains a reporter gene expression-cassette in antisense orientation; the reporter gene (either luciferase or YFP) is interrupted by an intron that is oriented in the sense direction. Reporter gene expression is dependent on splicing and packaging of the vector RNA in the producer cell and reverse transcription, integration and expression in the target cell. The HTLV-1 vector system is functional and will enable the quantitative study of cell-to-cell infection. We have developed a cell culture system in which Jurkat T cells are transfected with HTLV-1 vectors and then cocultured with Raji cells. HTLV-1 infection and replication in this system is dependent on viral envelope, reverse transcriptase, and integrase proteins. Infection is blocked by RT inhibitors and by anti-HTLV-1 neutralizing antibodies. Intriguingly, efficient transduction is dependent on expression HTLV-1 Tax, a pleiotropic activator of cellular gene expression, even though the viral vectors are Tax-independent. We are currently examining the roles of cellular adhesion molecules, cytoskeleton, and signal transduction pathways in the cell-to-cell infection process.

POXVIRUS ENTRY/FUSION PROTEINS: TOO MUCH OF A GOOD THING?*

Tatiana G. Senkevich, Alan C. Townsley, Suany Ojeda, Erica Brown, and Bernard Moss

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Poxviruses are large DNA viruses that replicate entirely in the cytoplasm of infected cells. Until recently, little was known about their mode of entry into cells and no viral fusion protein had been identified. Using a combination of bioinformatics, genetics, electron microscopy and biochemistry, we discovered and characterized eight viral proteins that form a stable complex in the vaccinia virus membrane. The assembly was not detected in the absence of the viral membrane and repressed synthesis of any one of these proteins interfered with formation of the complex or destabilized it, suggesting interrelated subunit architecture. An additional membrane protein that interacts with the complex but is not required for its formation was found. The nine proteins are conserved in all poxviruses, have a single N- or C-terminal transmembrane domain, are non-glycosylated and except for one have at least two intramolecular disulfide bonds. Thus far, inducible conditional lethal null mutants have been made for eight of the nine proteins and each has the same phenotype under non-permissive conditions: assembly of normal looking virions that bind to cells but cannot enter them. In addition, cells infected with any one of the conditional lethal mutants are unable to form syncytia following brief low pH treatment. Cell entry of vaccinia virus can occur either by fusion at the plasma membrane or by low pH-dependent endocytosis. However, the entry/fusion complex is required for either pathway. Elucidation of the mechanism of fusion and the roles of the individual proteins are under investigation.

*From Shakespeare's As You Like It.
ROSALIND: Why then, can one desire too much of a good thing?

HIV REPLICATION AND EVOLUTION IN PATIENTS ON HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

Robert F. Siliciano

Johns Hopkins University School of Medicine and Howard Hughes Medical Institute, Baltimore, MD

Treatment of HIV-1 infection with highly active antiretroviral therapy (HAART) reduces viremia to below the detection limit of ultrasensitive clinical assays. However, HIV-1 persists in resting $CD4^+$ T cells and possibly other reservoirs. In patients on HAART, HIV-1 persistence is evidenced not only by the latent reservoir in resting CD4+ T cells but also by free virus in the plasma. Given the short half-life of free virus, this residual viremia indicates active virus production. This virus production may reflect low-level ongoing replication that continues despite HAART and/or release of virus from latently infected cells that become activated or from other stable cellular reservoirs. With respect to the issue of drug resistance, it is of particular importance to determine whether ongoing replication continues on HAART because the evolution of resistance can only take place if there are new cycles of replication. The direct characterization of residual viremia provides a window into this state of virologic suppression and a means for determining the importance of different mechanisms of viral persistence. Characterization of this residual viremia has been limited because of the technical difficulties involved in the analysis of extremely low numbers of viral RNA templates. To obtain sufficient numbers of independent viral clones from the plasma of patients on HAART, we have carried out intensive sampling in a series of patients and analyzed plasma virus genotypes with a sensitive RT-PCR method. Viral variants in the plasma were compared to viruses in the latent reservoir. The results provided evidence that in some patients on HAART, much of the residual viremia is due to continued production of a small number of viral clones over prolonged periods without evident sequence change by cells that are not well represented in the circulation. The static nature of the residual viremia is not consistent with ongoing viral replication. These results have implications for understanding HIV-1 persistence and treatment failure.

HIV-1 EVOLUTION IN PATIENTS INFECTED WITH SINGLE OR MULTIPLE VARIANTS

M. Kearney[1,2], W. Shao[1], F. Maldarelli[1], J. Margolick[3], E. Daar[4], J. Mellors[5], V. Rao[2], J. Coffin[1] and S. Palmer[1]

[1]HIV Drug Resistance Program, NCI, NIH, [2]Catholic University of America, Washington, D.C., [3]Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, [4]Division of HIV Medicine Harbor-UCLA Medical Center, Los Angeles, CA, [5]Div. Inf. Dis., U. Pittsburgh, Pittsburgh, PA

Background: Knowledge of genetic diversity early in HIV-1 infection is critical for HIV-1 vaccine design. It is not clear whether diversity in acute infection arises from transmission of single or multiple HIV-1 variants. We therefore studied HIV-1 sequences in longitudinal plasma samples from acutely or recently infected, treatment-naïve patients.

Methods: Longitudinal plasma samples (n=83) were obtained from 11 patients with HIV-1 (subtype B) infection, 3 with acute infection (<30 days post-seroconversion) and 8 with recent infection (1-9 months post-seroconversion). Approximately 20 single genome sequences were obtained from two gene regions, *pro-pol* and *env*, for each sample. Genetic diversity was calculated as average pairwise differences (APD) and sequence divergence over time was assessed by phylogenetic analyses.

Results: The first available sample after infection in 10 of 11 patients revealed HIV-1 populations with small genetic diversities (APD ranging 0.01%–1.0% in *pro-pol* and 0.1–1.5% in *env*). The virus diversity in these 10 patients increased over time (6 months–5 year follow up) to as high as 1.5% in *pro-pol* and 4.5% in *env*. The exception (patient 11) showed 1.9% diversity in *pro-pol* and 5.4% diversity in *env* in a sample collected 13 days after a negative ELISA. Phylogenetic analyses of sequences from this patient suggest that infection resulted from transmission of at least 3 genetically distinct HIV-1 variants from a single donor, followed by further diversification and recombination among the transmitted variants. Longitudinal analysis of sequences in this patient showed that diversity decreased over a 4 month period from 1.9% to 0.4% in *pro-pol* while increasing in *env* from 5.4% to 7.5%. The decrease in *pro-pol* diversity in patient 11 appeared to result from the selective loss of *pro-pol* sequences from at least one of the transmitted variants. By contrast, longitudinal *env* sequences showed persistence of all transmitted variants with a greater sequence diversity compared to the other patients.

Conclusions: These data indicate that acute HIV-1 (subtype B) infection usually results from transmission and amplification of a single viral variant producing a homogeneous virus population that diversifies slowly. The transmission of multiple HIV-1 variants appears to be uncommon but can result in a diverse virus population in acute infection that persists in *env.* The mechanisms underlying single vs. multiple variant transmission remain undefined.

A MACAQUE MODEL TO STUDY HIV-1 DRUG RESISTANCE

Zandrea Ambrose[1], Sarah Palmer[1], Valerie Boltz[1], Mary Kearney[1], Kelli Oswald[2], Kay Larsen[3], Patricia Firpo[3], Leon Flanary[3], Frank Maldarelli[1], Stephen H. Hughes[1], Jason Kimata[4], John W. Mellors[5], Shiu-Lok Hu[3], John M. Coffin[1], Jeffrey D. Lifson[2], and Vineet N. KewalRamani[1]

[1]HIV Drug Resistance Program, National Cancer Institute, Frederick, MD; [2]AIDS Vaccine Program, SAIC-Frederick, Inc, National Cancer Institute, Frederick, MD; [3]Washington National Primate Research Center, Seattle, WA; [4]Baylor College of Medicine, Houston, TX; [5]Department of Medicine, University of Pittsburgh, Pittsburgh, PA

HIV drug resistance is thought to have at least two origins: (a) preexisting resistant mutants at a low frequency that are selected by therapy and (b) suboptimal therapy that permits replication, evolution and selection of resistant mutants. Studying the mechanisms and anatomic origins of HIV drug resistance in humans is constrained by limited blood and tissue sampling and ethical restrictions on therapy. Here we examined the impact of short-course efavirenz (EFV) monotherapy on the evolution of drug resistance in a macaque model of antiretroviral therapy. Six macaques were infected with a pathogenic SIV/HIV chimeric virus, RT-SHIV$_{mne}$, in which SIV$_{mne}$ RT is replaced with HIV-1 RT. Plasma viral loads were allowed to stabilize for 13 weeks before the animals received either 3 days of EFV or no treatment. Starting 4 weeks later, all animals received daily therapy with tenofovir (TDF), FTC, and EFV for 20 weeks, followed by treatment interruption. Resistance was measured by allele-specific PCR (ASP) and single genome sequencing (SGS). Triple drug therapy reduced plasma viral load to low or undetectable levels for 20 weeks in 5/6 animals. Viral rebound occurred after only 5 weeks of triple therapy in 1/3 animals who had received EFV monotherapy. ASP revealed NNRTI-resistant variants before triple therapy in 2/3 animals who had received EFV monotherapy of >20% frequency. Of note, the animal with failure of triple therapy had the highest plasma viral load setpoint (>10^6 copies/ml) before any therapy. SGS analyses revealed linkage of specific K103N and M184I/V resistance alleles during failure. To understand the effects of treatment interruption on selection of drug resistance, therapy was stopped and reinitiated after viral load rebound to above 3,000 copies vRNA/ml. In conclusion, we have shown that combination therapy with TDF/FTC/EFV was effective in suppressing RT-SHIV replication in 5/6 macaques. Brief exposure to EFV monotherapy resulted in selection of NNRTI-resistant variants in 2/3 animals and early failure of triple drug therapy in 1/3 animals. This new animal model of antiretroviral therapy has the flexibility to address many key questions about the selection, tissue origins and persistence of drug resistance that cannot be answered in human studies.

RESISTANCE ANALYSIS OF A POTENT NUCLEOSIDE INHIBITOR OF VIRAL REPLICATION IN A PRE-CLINICAL CHIMPANZEE STUDY OF CHRONIC HEPATITIS C INFECTION

Ludmerer S.W., Graham D., Davies M., Handt L., Koeplinger K., Zhang R., MacCoss M., Hazuda D., Carroll S.S., Olsen D.B.

Merck Research Labs, West Point, PA 19486

MK-0608 (2'-C-Me-7-deaza-adenosine) is a nucleoside inhibitor of the Hepatitis C virus NS5B polymerase (replicon EC_{50} = 0.3 μM) with robust activity (> 5 log drop in viral titer) in a chimpanzee model of infection. As part of its pre-clinical evaluation, infected chimpanzees dosed with MK-0608 were monitored for viral resistance. NS5B was cloned from viral RNA isolated from plasma samples collected prior to, during, and after MK-0608 dosing, and sequenced. Resistance mutations were identified within samples drawn from 2 of 6 chimpanzees. One chimpanzee dosed intravenously at 2 mg/kg per day had its viral load reduced from > 10^6 IU/ml to unquantifiable levels (BLQ, < 20 IU/mL) with subsequent rebound after dosing ceased. As virus levels began to rebound the sequences encoded a mixture of wild-type serine and a threonine mutation at residue 282. As viral load continued to rebound, the circulating virus reverted to a homogenous S282 population. In another study a chronically infected chimpanzee was dosed for 37 days at 1 mg/kg PO. Viral load quickly dropped to unquantifiable levels and remained BLQ throughout dosing and two weeks post-dosing before rebounding. Circulating virus from a post-dose BLQ sample demonstrated sequence heterogeneity at residue 282, with arginine, threonine, or isoleucine encoded. Within a week the viral load rebounded and was homogenous for S282. Although a 2nd chimpanzee in this study experienced a greater magnitude of viral load reduction (>5 log), the viral load never dropped below 10^2 IU/ml, and mutations at 282 were not observed at any time. The presence of mutations at residue 282 is consistent with earlier *in vitro* replicon studies demonstrating that MK-0608 resistance is mediated by an S282T mutation. The appearance of mutations only in low viral load samples from MK-0608 suppression and the rapid re-emergence of wild-type S282 upon removal of selective pressure suggests that 282T mutant virus is debilitated relative to wild-type and may not persist as more than a minute component of circulating viral populations in the absence of selective pressure.

HIV GAG UBIQUITINATION, ESCRT INTERACTION AND RELEASE

Sonja Welsch, Eva Gottwein, Stefanie Jaeger, Anja Habermann, Oliver T. Keppler and Hans-Georg Kräusslich

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Human immunodeficiency virus (HIV) is released by budding through a membrane of the infected cell. The viral Gag polyprotein is necessary and sufficient to induce release of virus-like particles. Ubiquitination of Gag as well as interaction of the C-terminal p6 domain of Gag with components of the ESCRT machinery has been implicated in HIV release, in strong analogy with formation of intralumenal vesicles at the limiting membrane of multivesicular bodies (MVB). HIV budding occurs at the plasma membrane in T cells and most cell lines, while morphogenesis has been reported to occur at MVB and late endosomes in primary human macrophages. Accordingly, ESCRT complexes are believed to be recruited to the plasma membrane in T cells, while being present at the budding compartment in macrophages.

We have analyzed the importance of ubiquitination by mutating potential acceptor sites in the C-terminal part of Gag. Cumulative eliminiation of lysine-residues downstream of the capsid (CA) domain caused a significant delay in virus budding, while mutation in single domains had little effect. Ubiquitination was dependent on virus assembly at a cellular membrane, while no specific Gag subdomain appeared to be needed. Immuno-EM analysis of the localization of several ESCRT-proteins indicated that they are distributed to endosomal membranes and to the plasma membrane, and no specific recruitment to the plasma membrane was observed in T cells or T cell lines. Lastly, we have re-assessed the budding compartment in infected primary human macrophages and suggest that HIV-1 budding occurs predominantly at the plasma membrane and plasma membrane invaginations also in these cells.

THE CONFORMATION OF THE HIV-1 GAG PROTEIN UNDERGOES A MAJOR CHANGE UPON VIRUS PARTICLE ASSEMBLY

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A single viral protein, termed Gag, is sufficient for assembly of retrovirus-like particles in mammalian cells. We have purified the HIV-1 Gag protein (lacking myristate at its N-terminus and the p6 domain at its C-terminus) from bacteria. This protein is capable of assembly into virus-like particles in a defined *in vitro* system. This protein is in monomer-dimer equilibrium in solution, but we identified a mutant protein (in which Trp184 and Met185 of the capsid domain are replaced with alanines) that remains monomeric except at very high concentrations. This mutation enabled us to analyze solutions of monomeric protein.

Hydrodynamic studies on the mutant protein showed that it is highly asymmetric or extended, with a frictional ratio of 1.66. Small-angle neutron scattering (SANS) experiments confirmed that it is highly asymmetric, with an R_g of 34 Å.

Atomic-level structures of individual domains within Gag have previously been determined, but these domains are connected in Gag by flexible linkers. We constructed a large series of models of the monomeric Gag protein by joining these domain structures together *in silico*, and tested each model computationally for its agreement with the experimental hydrodynamic and SANS data. The only models consistent with the data were those in which Gag was folded over, with its N-terminal matrix domain near its C-terminal nucleocapsid domain in 3-dimensional space.

Since Gag is a rod-shaped molecule in the assembled immature virion, these findings imply that Gag undergoes a major conformational change upon incorporation into the virus particle. Current work is directed toward identifying the factors responsible for this change.

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TARGETING VIRUS ASSEMBLY

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One approach to antiviral therapeutics ought to be interfering with the assembly of these complex biological machines. This is particularly attractive since a typical virus is comprised of 100s to 1000s of pieces. The problem with this approach is that identifying and designing small molecules that target protein-protein interactions is not particularly easy: there is no obvious transition state analog, no directly measurable enzymatic activity, and no simple generalizations about assembly-affecting binding sites. We will need to test whether assembly is indeed a practical target. The first point I want to make is that inhibiting assembly of infectious virions can be accomplished by **inhibiting or enhancing** assembly of viral core/capsid proteins. The studies I will talk about focus on hepatitis B virus, a distant relative of retroviruses, whose reverse transcription and trafficking are dependent upon correct core assembly. The capsid of HBV is assembled from 120 capsid protein dimers, arranged with T=4 quasi-symmetry. We have developed a fluorescence-based screen for molecules that affect assembly that can readily detect enhancement and inhibition of assembly. Capsid protein is C-terminally labeled with a fluorophore that self-quenches on assembly. We monitor changes in the extent and kinetics of assembly in a 96-well format. Once an assembly effector is identified its mechanism must be determined. We have worked extensively with HAP (heteroaryl dihydro pyrimidine) molecules, originally discovered by Bayer. We have shown that HAP1 accelerates assembly, destabilizes fivefolds, and increases the stability of quasi-sixfolds. HAP can favor assembly of icosahedral particles or aberrant structures, depending on concentration. A crystal structure of HBV capsids with and without HAP show that the small molecule acts as a molecular wedge that distorts quaternary structure without noticeably changing tertiary structure of the capsid protein. In agreement with solution studies, we find that HAP flattens quasi-sixfolds, making them more amenable to forming sheets, and bows fivefolds, changing the surface buried by protein-protein contacts.

USE OF HIGH RESOLUTION X-RAY CRYSTALLOGRAPHY TO ELUCIDATE THE MECHANISMS OF HIV-1 PROTEASE DRUG RESISTANCE

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Although HIV-1 protease (PR) inhibitors revolutionized the anti-AIDS therapies, drug resistance to the available clinical drugs is the principal concern during HAART. The study of the molecular basis of drug resistance is therefore of paramount importance, leading to the design of improved drugs that maintain their effectiveness against multi-drug resistant PR variants. The analysis of changes in PR/ligand interactions that occur due to mutations helps to understand the drug resistance properties of a mutant PR. Such detailed comparison of the frequently subtle structural differences requires high resolution structures to be available.

Here we present high resolution crystal structures and enzyme kinetics data of drug resistant PR variants PR_{V32I} and PR_{M46L} complexed with the FDA approved inhibitor darunavir (DRV). PR_{V32I}/DRV (0.84Å) and PR_{M46L}/DRV (1.22Å) structures show the inhibitor bound at two distinct sites, one in the active-site cavity and the second on the surface of the flexible flaps in the PR dimer. DRV binds at these two sites simultaneously in two diastereomers related by inversion of the sulfonamide nitrogen. Moreover, the flap site is shaped to accommodate the diastereomer with the *S*-enantiomeric nitrogen rather than the one with the *R*-enantiomeric nitrogen. The existence of the second binding site and two diastereomers suggest a mechanism for the high effectiveness of DRV on drug resistant HIV and the potential design of new inhibitors. In the enzyme kinetics experiments using UV absorbance assay, RP_{V32I} and PR_{M46L} showed similar or 50% activity of the PR_{WT}, respectively, and were 7 to 10 times more resistant to the inhibition by DRV. The inhibition kinetics data correlate well with the altered interactions in the mutant PR/DRV complexes compared to those in PR_{WT}/DRV. The implications for development of resistance to DRV are discussed.

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99 IS NOT ENOUGH: MOLECULAR CHARACTERISATION OF DRUG-RESISTANT HIV-PROTEASE MUTANTS WITH INSERTIONS IN THE FLAP REGION

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Amino acid insertions in the HIV reverse transcriptase (RT) are well established as a molecular mechanism of resistance against RT inhibitors. However, only few reports on insertions in the protease (PR) coding region localised in the external loops of the PR structure have been published. It is unclear whether or not the insertions are responsible for the resistant phenotype. We identified amino acid insertions in the positions 33 and 35 of HIV PR sequence of a patient treated by protease inhibitors for a prolonged period of time and set out to characterize the contribution of these insertions to the viral resistancy on the molecular level.

Resistant PR variants with or without the insertions in the positions 33 and 35 were cloned in *E.coli*, purified and characterised using chromogenic peptide substrate and a panel of inhibitors. X-ray structures of the resistant PR-inhibitor complexes were determined to 1.8 A resolution with very good structural factors.The E35EE insertion does not significantly decrease the catalytic activity of the enzyme but brings about approximately tenfold increase in the relative inhibition constant for lopinavir and other PIs. The X-ray structure analyses of the mutated proteases suggest enlargement of the PR binding site and changes in the dynamics of the flap movement as the cause for the weaker binding of the inhibitor to the PR binding site. We conclude that amino acid insertions into the PR sequence in the vicinity of the binding cleft represent novel mechanism of HIV resistance development.

CO-EVOLUTION OF HIV-1 PROTEASE AND SUBSTRATES IN RESPONSE TO INHIBITOR THERAPY - AN INVESTIGATION OF NOVEL SITES

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HIV-1 protease and occasionally its substrates evolve to cause drug resistance under selective pressure of the competitive protease inhibitors. Co-evolution of Gag and Gag-Pol substrate cleavage sites may occur to compensate for any changes in protease activity. However, studies have shown that not all substrates co-evolve. We hypothesize that cleavage sites co-evolve only when they extend beyond the consensus "substrate envelope" to contact residues that mutate in response to protease inhibitors. In this study we focus on changes in the cleavage sites sequences that co-evolve with particular drug resistant protease variants. Sequence data obtained from samples submitted for routine resistance testing (Monogram Biosciences) were analyzed for correlated changes in protease and its substrates using Chi-square analysis. The phi correlation coefficients were also calculated to classify correlations as positive or negative. Modeling studies were performed using PyMOL to investigate structural changes that may occur as a result of these correlated mutations. We observed three novel sites of co-evolution within the cleavage sites that we analyzed. Correlations were observed between 1) V82A in protease and the PR-RT cleavage site 2) I50V in protease and PR-RT cleavage site and 3) I50L in protease and NC-p1 cleavage site. We also confirmed earlier reported co-evolution of the p1-p6 cleavage site with either D30N/N88D or I50V in protease. We are currently analyzing the structural basis for this specificity. Co-evolution of cleavage site substrates in the Gag and GagPol polyproteins with resistance mutations in HIV-1 protease appears to be more wide-spread than previously reported. These observations suggest further avenues of experimentation to more fully understand the potential impact of gag and gag-pol cleavage sites mutations on drug susceptibility and protease inhibitor resistant virus fitness.

CHARACTERIZATION OF V32I/I47A HIV-1 PROTEASE MUTATIONS CONFERRING A HIGH LEVEL RESISTANCE TO LOPINAVIR

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Selection of resistance to first-line lopinavir/ritonavir (LPV/r) therapy has not been documented thus far. Multiple protease (PR) mutations in protease inhibitor (PI)-experienced patients are necessary for high-level phenotypic resistance to LPV with the exception of a rare emergence of I47A genotypic change in PR that has been specifically associated with LPV/r treatment (de Mendoza, AIDS, 2006). In this study, we identified and characterized a V32I/I47A PR mutant selected *in vitro* in the presence of LPV.

The exposure of the HIV-1 IIIB strain to LPV for >6 months selected for a variant with L10F/M46I/I84V mutations in PR and 10- to 25-fold reduced susceptibility to LPV and multiple other PIs. In contrast, parallel selection with the 89.6 strain resulted in V32I/I47A mutations in PR with no changes identified in Gag. The selected virus showed 160-fold resistance to LPV, 10- to 20-fold reduced susceptibility to amprenavir (APV) and several other PIs, and a slight hypersusceptibility to saquinavir (SQV). Similar to the LPV-selected 89.6 variant, site-directed recombinant virus with V32I/I47A was resistant to LPV and hypersensitive to SQV. The V32I/I47A enzyme exhibited 600- and 300-fold elevated K_i for LPV and APV, respectively, relative to the wild-type PR. Interestingly, the mutant PR had 1.6-fold lower K_i value for SQV than wild-type PR. Data from isothermal titration calorimetry revealed minimal changes in LPV binding entropy ($-T\Delta S$), but a substantial loss (>5 kcal/mol) in the enthalpic component (ΔH) of the LPV binding energy due to V32I/I47A mutations, resulting in >2,000-fold reduction in the inhibitor binding affinity (K_d). Structural modeling indicates that the reduced ΔH is primarily due to a loss of van der Waals contacts between residues 47/47' and LPV in the mutant PR. This does not occur with SQV because of its minimal contacts with residues 47/47'.

In conclusion, the *in vitro* selected variant of HIV-1 89.6 with V32I/I47A mutations in PR showed markedly reduced susceptibility to LPV compared to other PIs. This high-level resistance is a consequence of the diminished affinity of LPV for the mutant enzyme due to a dramatic change in the inhibitor binding enthalpy.

INHIBITION OF EXOGENOUS AND ENDOGENOUS GENETIC ELEMENTS BY APOBEC3 CYTIDINE DEAMINASES

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APOBEC3 proteins constitute a family of cytidine deaminases that provide intracellular resistance to retroviruses and interfere with the transposition of endogenous retroelements. The various members of the family are cytidine deaminases but vary in their specificity for various genetic elements. APOBEC3G is particularly active against HIV-1 but weakly active against LTR retroelements. APOBEC3B is only slightly active against HIV-1 but potently inhibits SIV. APOBEC3A (hA3A) is particularly interesting because it is inactive against HIV-1 and SIV but potently inhibits LTR and non-LTR retroelements. It has a single active site and is catalytically active on single-stranded DNA. Interestingly, hA3A is highly effective against the parvovirus adeno-associated virus (AAV), a small eukaryotic DNA virus that replicates in the nucleus through a single-strand intermediate. hA3A is also a potent inhibitor of the endogenous LTR retroelements, MusD, IAP and the non-LTR retroelement, LINE-1. hA3A function is dependent on the conserved amino acids of the deaminase active site suggesting a role for cytidine deamination. However, mutations in retroelement sequences were not found. hA3A is localized in both the cytoplasm and nucleus which may contribute to its unique ability to inhibit AAV and retrotransposons. Analysis of the dual active site APOBEC3 proteins showed that for the human proteins, only the second domain is catalytically active. In contrast, the arrangement is reversed for mouse APOBEC3 where domain 1 is catalytic and domain 2 mediates virion packaging. The APOBEC3 family members have distinct functions and may have evolved to resist various classes of genetic elements.

APOBEC3G INHIBITS HIV-1 DNA REPLICATION AND INTEGRATION

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Encapsidation of host restriction factor APOBEC3G (A3G) into Vif-deficient HIV-1 blocks virus replication at least partly by C-to-U deamination of viral minus-strand DNA, resulting in G-to-A hypermutation. A3G may also inhibit HIV-1 DNA replication by reducing viral DNA synthesis or inducing its degradation. To gain further insight into the mechanisms of viral inhibition, we examined the metabolism of A3G-exposed viral DNA. We observed that an overall 37-fold decrease in viral infectivity was accompanied by a sixfold reduction in viral DNA accumulation; wild-type A3G inhibited integration an additional fourfold in cells and similarly inhibited integration activity of HIV-1 preintegration complexes in vitro. A3G C-terminal catalytic domain was required for both of these antiviral activities. These data reveal that inhibition of viral DNA replication and integration are two additional and novel mechanisms by which A3G can mediate retrovirus restriction.

MASS SPECTROMETRY ANALYSIS OF HIV-1 VIF REVEALS AN INCREASE IN ORDERED STRUCTURE UPON OLIGOMERIZATION IN REGIONS NECESSARY FOR VIRAL INFECTIVITY

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HIV-1 Vif performs an important role in viral pathogenesis by facilitating the degradation of APOBEC3G, an endogenous cellular inhibitor of HIV-1 replication, through interactions with Cullin5 and elongin C in a cullin RING ligase (CRL) complex. In addition to the CRL proteins, HIV-1 Vif binds a host of other macromolecules including HIV-1 Gag and HIV-1 viral RNA. In this study, intrinsically disordered regions are predicted in HIV-1 Vif using sequence based algorithms. These regions of disorder may give insight into why traditional structure determination of HIV-1 Vif has been elusive. Because of the inability to perform traditional structure determination, chemical cross-linking along with ^{18}O labeling, proteolysis, and mass spectrometry was used to determine HIV-1 Vif's structural topology and to map the oligomerization domain. Cross-linking showed evidence of monomer, dimer, and trimer species via denaturing gel analysis and an additional tetramer via western blot analysis. Between the four samples: (1) noncross-linked and (2) cross-linked monomer, (3) dimer, and (4) trimer, 47 unique linear peptides and 24 (13 intramolecular; 11 intermolecular) noncontiguous, cross-linked peptides were identified. The peptide coverage reveals almost complete coverage of the N-terminus in all samples analyzed, but reduced coverage in the C-terminal region of the dimer and trimer samples. These differences in peptide coverage or "protections" between dimer and trimer indicate specific differential packaging between the 2 oligomeric forms. Intramolecular cross-links within the monomer suggest the N-terminus is likely folded into a compact domain, while the C-terminus remains intrinsically disordered. Intermolecular cross-links observed in the dimer and trimer species suggest the C-terminus undergoes a disorder-to-order transition upon oligomerization. In addition, 27 residues identified via mass spectrometry as being structurally important overlap with 29 residues previously shown to be important for viral infectivity. Furthermore, these "protections" and cross-links are consistent with the proposed oligomerization domain, Cullin5, elongin C, HIV-1 Gag, and HIV-1 viral RNA binding, thus highlighting these regions as "hot spots" for biological activity. Therefore, the biologically functional form of HIV-1 Vif likely involves a dynamic equilibrium between various oligomers, and the disorder-to-order transition observed in the C-terminus likely allows HIV-1 Vif to interact with other binding partners.

HIV-1(DELTA-VIF) CAN RESIST APOBEC3G

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APOBEC3G is an anti-retroviral cellular enzyme that can inhibit the replication of Vif-deficient HIV-1 by hypermutating nascent cDNA. In a normal infection, Vif functions to effectively neutralize APOBEC3G by marking it for degradation. Nevertheless, the remarkable potency of APOBEC3G has encouraged the development of therapeutic strategies that would prevent its neutralization. For instance, compounds that inhibit Vif would theoretically render the virus sensitive to APOBEC3G. If such drugs were to be discovered, an important question is whether such a 'Vif-deficient' virus might ultimately be able to evolve to resist APOBEC3G (of course, in addition to the compound itself)?

To begin to answer this question, APOBEC3G was expressed stably and constitutively in a T cell line and HIV growth experiments were performed. The parental cell line was fully permissive for HIV(delta-Vif) growth, whereas the APOBEC3G-expressing derivatives were completely non-permissive. Vif-proficient virus was able to replicate efficiently on both the parental and clonal lines with replication peaking approximately 7-10 days post-infection. In most continuous culture experiments, HIV(delta-Vif) failed to replicate on the APOBEC3G-expressing clones. However, after more than 28 days of continuous culture, a sizable fraction of the APOBEC3G-expressing cultures showed signs of HIV(delta-Vif) replication suggesting the emergence of APOBEC3G-resistant virus. This possibility was confirmed using cell-free supernatants to infect new APOBEC3G-expressing cells. Robust replication of the resulting virus was observed within 15 days. The molecular nature of some of the mutations that confer resistance to APOBEC3G and how these studies impact our current understanding of the APOBEC3G-dependent restriction mechanism will be discussed.

EVOLUTIONARILY CONSERVED AND NON-CONSERVED RETROVIRUS RESTRICTION ACTIVITIES OF ARTIODACTYL APOBEC3F PROTEINS

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The APOBEC3 proteins are unique to mammals. Many inhibit retrovirus infection through a cDNA cytosine deamination mechanism. HIV-1 neutralizes this host defense through Vif, which triggers APOBEC3 ubiquitination and degradation. Here, we report an APOBEC3F-like, double deaminase domain protein from three artiodactyls: cattle, pigs and sheep. Like their human counterparts, APOBEC3F and APOBEC3G, the artiodactyl APOBEC3F proteins are DNA cytosine deaminases that locate predominantly to the cytosol and can inhibit the replication of HIV-1 and MLV. Retrovirus restriction is attributable to deaminase-dependent and -independent mechanisms, as deaminase-defective mutants still retain significant anti-retroviral activity. However, unlike human APOBEC3F and APOBEC3G, the artiodactyl APOBEC3F proteins have an active amino terminal DNA cytosine deaminase domain, which elicits a broader dinucleotide deamination preference, and they are resistant to HIV-1 Vif. These data indicate that DNA cytosine deamination, subcellular localization and retrovirus restriction activities are conserved in mammals, whereas active site location, local mutational preferences and Vif susceptibility are not. Together, these studies indicate that some properties of the mammal-specific, APOBEC3-dependent retroelement restriction system are necessary and conserved, but others are simultaneously modular and highly adaptable.

CYCLOPHILIN, TRIM5, AND INNATE RESISTANCE TO HIV-1

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The peptidyl-prolyl isomerase cyclophilin A (CypA) binds an exposed, proline-rich loop on HIV-1 capsid (CA) and acts after virion membrane fusion with human cells to increase HIV-1 infectivity. HIV-1 CA is similarly greeted by CypA soon after entry into non-human primate cells, where, paradoxically, the interaction decreases HIV-1 infectivity. Attempts to understand the effects of CypA on HIV-1 infectivity, and independent efforts to understand the block to HIV-1 infection in old world primates, each led to the discovery of TRIM5, a gene that confers innate resistance to retroviral infection in primates. TRIM5 specificity in owl monkeys is conferred by a C-terminal CypA domain that binds CA of HIV-1, SIVAGMtantalus, and FIV. In other primate species, specificity for the CA of particular retroviruses is conferred by the C-terminal SPRY domain. TRIM5 then, may be thought of as a cytoplasmic receptor within the innate immune system which recognizes CA-specific determinants on the retroviral protein core. We will present data from ongoing experiments concerning how TRIM5 recognizes CA and concerning the mechanism by which TRIM5 restricts retroviruses.

PATHWAYS OF LEAST RESISTANCE: EFFECTS OF INTEGRASE MUTATIONS ON INHIBITORS OF HIV-1 INTEGRASE STRAND TRANSFER

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Inhibitors of the integrase strand transfer reaction have proven to be effective inhibitors of HIV-1 replication in vitro and in vivo, both in SIV-1 infected rhesus macaques and in HIV-1 infected patients. We have previously reported that in cell culture structurally distinct strand transfer inhibitors can select for unique mutations which selectively confer resistance to these compounds when introduced into the virus. These results have suggested it may be possible to identify integrase inhibitors with minimally overlapping resistance. To understand the extent of cross resistance among different chemical classes of strand transfer inhibitors we selected resistant variants using a variety of structurally distinct compounds (including MK-0518) and profiled large numbers of inhibitors against a panel of engineered viruses which encompass all of the integrase mutations observed in these studies as well as published reports. Although some integrase inhibitors were affected by mutations at any of several different residues, we observed that these compounds preferentially selected for those mutations which confer the least impact on viral replication fitness. Within a given chemical series, it was possible to identify integrase inhibitors which displayed significant differences in their respective profiles, however many compounds from different structural classes exhibited overlapping resistance patterns suggesting the potential for substantial cross resistance. The two compounds currently in clinical development MK-0518 and JT-303/GS9137 are from two distinct chemical series but displayed overlapping resistance profiles in these analyses. These studies suggest MK-0518 and JT303/GS9137 have a high potential to select HIV-1 variants that would be cross resistance in the clinic.

DISSECTING HIV-1 INTEGRASE CONTACTS WITH VIRAL DNA ENDS

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To identify HIV-1 integrase (IN) contacts with LTR ends we employed a new experimental strategy combining the two established methodologies of site specific protein-DNA cross-linking through disulfide bond formation and mass spectrometric protein foot-printing. Two mutant proteins, E152C and S230C, were first cross-linked to their respective sites (2nd and 7th nucleotides) on the DNA substrate. Surface topologies of the free protein and two IN-DNA complexes were next compared using lysine and arginine specific small chemical modifiers. Importantly, this approach enabled us to delineate monomer selective contacts with DNA substrate. In particular, we observed that in the E152C-DNA(2) complex certain core and N-terminal domain amino acids, and not C-terminal residues were shielded by interactions with DNA. In the S230C-DNA(7) complex certain C-terminal domain amino acids and not N-terminal or core domain residues were protected from the modification by DNA contacts. Site directed mutagenesis studies confirmed the functional importance of the identified residues. These findings indicate that N-terminal and core domains of the same monomer bind DNA substrate, while C-terminal domain of the separate monomer provides essential complementary contacts to DNA. Our data provide new and important details on full length HIV-1 IN interactions with LTR ends and enable plausible molecular modeling of the functional nucleoprotein complex. The experimental approach described herein has a generic application for mapping protein-nucleic acid contacts.

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VIRUS EVOLUTION REVEALS AN EXCLUSIVE ROLE FOR LEDGF/P75 IN HIV REPLICATION

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Retroviruses per definition insert their viral genome into the host cell chromosome. Although the key player of retroviral integration is viral integrase, a role for cellular cofactors has been proposed. The interplay between host proteins and HIV is however poorly understood. Lentiviral integrases use the cellular protein LEDGF/p75 to tether the preintegration complex to the chromosome. Truncation mutants of LEDGF/p75 lacking the chromosome attachment site strongly inhibit HIV replication by competing for the interaction with integrase. Using this approach we selected different HIV strains that can overcome this inhibition. Detection of integrase mutations at key positions in the LEDGF/p75-integrase interface provides critical evidence of the importance of LEDGF/p75 in HIV integration. The data provide a striking example of the power of viral molecular evolution. In spite of resistance development, the affinity of integrase for LEDGF/p75 is reduced and replication kinetics is impaired. Moreover, the complementary inhibition by LEDGF/p75 knockdown and mutagenesis at the integrase-LEDGF/p75 interface points to the incapability of HIV to circumvent LEDGF/p75 function during proviral integration. Together these data support the integrase-LEDGF/p75 interaction as a potent, new antiviral target.

HIV REV AS A THERAPEUTIC TARGET

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The Rev protein is absolutely essential for HIV replication, since in the absence of Rev, genomic RNA and several other HIV mRNAs cannot exit the nucleus. Thus, when Rev is inhibited, viral structural proteins are not made and the infectious cycle cannot continue. Since many of the interactions that occur in the Rev export pathway are completely viral in nature, specific targeting of these interactions by a therapeutic agent would be expected to lead to specific inhibition of viral replication with potentially only minimal side effects on cellular functions.

A cell-based screening assay was used to screen a library of 40,000 compounds and two small molecules were identified that appear to inhibit HIV replication by interfering with Rev function. They are active at uM concentrations, and have therapeutic indices of at least two logs in most of the assays they have been tested in. They do not inhibit Rev-RRE binding *in vitro*. However, the compounds show good specificity for Rev inhibition, since they inhibit virus particle formation when it is driven by the Rev-RRE pathway, but not when it is driven by the CTE pathway. In addition, the ratio of Rev-dependent gene products to Rev-independent gene products is greatly reduced in infected cells that are treated with the compounds.

Viruses resistant to two of the compounds were isolated by long term culture in the presence of suboptimal concentrations of compound. Resistant variants demonstrated cross-resistance to both compounds. DNA sequencing identified mutations in the RRE that were shown to convey the resistant phenotype. Interestingly, these mutations map to the Stem Loop IIc region of the RRE, which is adjacent to the primary Rev binding site.

These compounds are thus promising leads as therapeutic candidates that target HIV replication through inhibition of Rev function and may, in addition, be useful tools in for the elucidation of the Rev-RRE pathway. They also validate the notion of Rev as therapeutic target worthy of further development and study.

N-TERMINAL FRAGMENT OF EIF3P47 INHIBITS HIV-1 INFECTION

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Cells have evolved multiple defense mechanisms to inhibit retroviral replication. Host susceptibility is limited by the expression of dominant factors that may restrict multiple steps in viral replication. We have taken a general approach to identify new molecules in human cells that are capable of restricting HIV replication. We screened a mammalian complementary DNA (cDNA) library for genes that prevent infection by genetically marked retroviruses. This library was introduced into a human virus susceptible cell line, and a virus resistant population was selected for survival after multiple rounds of infection with an HIV particle expressing a toxic gene delivered by the pantropic envelope VSV-G. Two cellular clones were recovered in this selection; we will be focusing on one of the clones dubbed TE.H2. Compared to the parental cell line TE671, TE.H2 was 100 times less sensitive to infection by an HIV vector expressing the puromycin resistance gene (HIV-Puro/VSV-G) and 5 times less sensitive to MLV-puro/VSV-G infection. To distinguish whether HIV resistance was accorded by the cDNA present in these clones or due to selective outgrowth of spontaneous mutations, the cDNA recovered from clone TE.H2 was reintroduced in fresh TE671 cells and was shown to induce a similar resistance to infection as TE.H2. Transfection and real time PCR experiments results suggest that this cDNA expression did not interfere with early events but rather inhibited retroviral gene expression; proviruses are formed normally but viral mRNA levels were reduced in the cytoplasm of resistant TE.H2 cells upon infection. The cDNA recovered from TE.H2 cells encodes the N-terminal portion (91 a.a.) of the eukaryotic initiation factor 3 subunit p47, eIF3p47. The replication of wild type HIV particles is severely restricted in cells expressing this cDNA. Understanding the mechanism by which this factor interferes with HIV infection may provide insight into post-integration steps in the HIV replication cycle.

ELEMENTS OF THE NUCLEAR TRANSPORT MECHANISM AS ANTIVIRAL DRUG TARGETS: OVEREXPRESSION OF THE CONSERVED DOMAINS OF A FISSION YEAST NUCLEOPORIN SELECTIVELY KNOCKS DOWN RETROTRANSPOSITION WITHOUT AFFECTING ITS HOST

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The nucleoporin Nup124p[1] is a host protein required for the nuclear import of both, retrotransposon Tf1-Gag as well as the retroviral HIV-1 Vpr[2] in fission yeast. The human nucleoporin Nup153 and the *S. cerevisiae* Nup1p were identified as orthologs of Nup124p[2]. Nup124p and Nup153 share a unique N-terminal domain[2], whereas all three nucleoporins share a large FG/FXFG-repeat domain and a C-terminal peptide sequence- xRKIxxxxxRRKRx(R) that are absolutely essential for Tf1 retrotransposition. In this study, the contributions of the FG/FXFG-repeat domain and C-terminal peptide sequence to Tf1 transposition were evaluated by mutational analysis, domain-swapping experiments and other biochemical and genetic tests. Interestingly, though the FXFG domain was essential, the FXFG-repeats themselves could be eliminated without loss of Nup-mediated viral activity suggesting the existence of a common element unrelated to FG/FXFG motifs. The Nup124p C-terminal peptide-GRKIAVPRSRRKR was extremely sensitive to certain single amino acid changes within stretches of the basic residues. Based on our comparative study of Nup124p, Nup1p and Nup153 domains, we have developed peptides that specifically knockdown viral activity by disengaging the virus from its host nuclear transport machinery and without any harmful consequence to the host itself. Our results imply that those domains challenged a specific pathway affecting Tf1 transposition. Although full-length Nup1p or Nup153 does not complement Nup124p, their *modus operandi* with reference to Tf1 activity suggest that these three proteins evolved from a common ancestor. Thus, the study of common domains affecting nucleoporin-mediated viral activity holds the promise of selectively blocking nuclear transport of viral material and as a consequence, the development and design of antiviral drugs.

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NOVEL MECHANISM OF RESISTANCE TO AN HIV-1 ENTRY INHIBITOR: CLEAVAGE OF THE GP41 CYTOPLASMIC TAIL BY THE VIRAL PROTEASE

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Membrane cholesterol plays an important role in HIV-1 assembly and infectivity. We investigated the target and mechanism of action of the cholesterol-binding compound amphotericin B methyl ester (AME), a water-soluble, relatively non-toxic derivative of the polyene fungal antibiotic amphotericin B. We observed that AME potently inhibits the replication of HIV-1 in various T-cell lines and in primary lymphocytes and macrophages. By analyzing the effect of AME on ~25 strains of HIV-1, we demonstrate that AME blocks the replication of diverse HIV-1 isolates, irrespective of their clade, target cell tropism, or resistance to RT or PR inhibitors. We show that AME profoundly impairs virus entry and also induces a ~5-fold decrease in virus particle production.

To study further the antiviral properties of AME, we selected for AME-resistant virus in Jurkat T-cells. We observed that mutations responsible for AME resistance (P203L and S205L) mapped to an endocytosis motif in the cytoplasmic tail of gp41. Virus replication and single-cycle infectivity assays confirmed that the P203L and S205L substitutions confer AME resistance. Interestingly, truncation of the cytoplasmic tail of either HIV-1 or SIVmac gp41 also renders these primate lentiviruses resistant to AME. We investigated the connection between point mutations in the gp41 cytoplasmic tail and gp41 truncation. Remarkably, examination of gp41 in virions bearing the P203L and S205L mutations revealed the presence of a truncated gp41 species. Further analysis demonstrated that the P203L and S205L mutations induce the cleavage of the gp41 cytoplasmic tail by the viral protease. Thus, in response to inhibition by AME, HIV-1 has evolved a strategy used by murine leukemia virus and Mason-Pfizer monkey virus: retain the TM tail until Env has been incorporated, then cleave off the tail with the viral protease to activate fusogencity. This study identifies a novel mechanism of resistance used by HIV-1 to escape from an entry inhibitor.

ANTIVIRAL STRATEGIES FOR DISRUPTING POXVIRUS REPLICATION: LISTENING TO CUES FROM THE VIRUS AND THE CELL

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Poxviruses are complex DNA viruses that replicate solely in the cytoplasm of infected cells, and therefore display an unusual degree of physical and genetic autonomy form the host. Molluscum contagiosum, monkeypox virus and variola, the causative agent of smallpox, are poxviruses associated with human disease. Vaccinia virus is the prototypic poxvirus for experimental research, as well as serving as the vaccine administered for protection against variola infection. Our laboratory has been involved in characterizing both the *cis* and *trans* acting components of the replication machinery. Essential proteins involved in replication include the catalytic polymerase (E9), the A20 protein, the D4 protein, an ATPase with homology to superfamily III helicases (D5), and a protein kinase (B1). We will address several facets of viral replication. First, we have recently determined that the processive holoenzyme that directs genome replication is comprised of the catalytic subunit of the enzyme (E9 protein) in association with a stable heterodimeric complex containing the A20 protein and the viral uracil DNA glycosylase (UDG, D4 protein). We are investigating the possibility that compounds able to interfere with the A20:UDG interaction would be effective inhibitors of poxvirus replication.

Secondly, we will discuss our new findings regarding the role of the B1 protein kinase. The precise role for B1 in regulating DNA replication has been difficult to assess, since it does not appear to modify any of the other replicative functions. B1 shows significant homology to a family of cellular protein kinases named vaccinia related kinases 1, 2 and 3 (VRKs). Indeed, catalytically active VRK1 can complement the replicative defect seen in a *ts*B1 infection. We have determined that both B1 and VRK1 can rapidly and efficiently phosphorylate the cellular BAF protein. BAF is a ubiquitous protein found in both the nucleus and cytoplasm; in the nucleus, it associates with both chromatin and proteins of the inner nuclear membrane and is thought to play important roles in the disassembly and reassembly of the nuclear membrane during mitosis. Cytoplasmic BAF is exploited by retroviruses; BAF binds to pre-integration complexes and has been reported to prevent proviral auto-integration and facilitate proviral integration into the host chromosome. These roles for BAF rely on its ability to bind double-stranded DNA with high affinity and without sequence specificity. We have defined the residues on which VRK1 and B1 phosphorylate BAF, and have shown that modification of these residues eliminates DNA binding activity.

We have shown that cytoplasmic BAF plays an important role as a cellular defense against poxvirus replication. In the absence of active B1, cytoplasmic BAF associates with viral replication factories in a manner that correlates with the cessation of DNA synthesis. When B1 is active, BAF remains diffuse. Expression of BAF from the viral genome is tolerated, but expression of a non-phosphorylatable derivative of BAF is lethal. When cell lines engineered to overexpress BAF are infected with *ts*B1, the temperature-sensitive defect is exacerbated. In contrast, when infections are performed in cells in which the cytoplasmic pool of BAF has been largely depleted, the *ts* defect is largely overcome. Thus, the primary role of the poxvirus B1 enzyme is to combat the ability of BAF to bind to the cytoplasmic viral genome and sequester it in a fashion that is incompatible with replication. We propose that BAF serves as a sensor of exogenous ,cytoplasmic DNA, and as such is an effector of a novel innate immune defense.

A YEAST TRANSPOSON SERVES AS A MODEL FOR HIV-1 INTEGRATION

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The retroviruses HIV-1 and MLV integrate preferentially into genes transcribed by pol II. The LTR-retrotransposon, Tf1 found in *Schizosaccharomyces pombe* also integrates into pol II transcribed genes. But in this case, the insertions occur specifically at the promoter regions. Because the integration preference of retroviruses for pol II transcribed genes is not yet understood, Tf1 is an excellent model for studying this pattern. To understand what factors contribute to the selection of target sites, we developed a plasmid-based method for detecting multiple insertions in target sequences. Target plasmids were introduced into strains of *S. pombe* that were then induced for Tf1 transposition. We tested whether the two divergent promoters between *bub1* and *ade6* were targets of integration. Of 43 insertions in the target plasmid, 41 (95%) occurred within a 160nt window of the promoter region. Deletion of either the *bub1* or *ade6* ORFs did not reduce targeting of the promoter. Similarly, deletions that removed DNA upstream and downstream of the target window in the promoter region did not alter the insertion pattern or efficiency. However, deletion of the exact region of integration caused a significant reduction of insertions in the target plasmid. Data from RNA blots demonstrated that deletions in the promoter region could lower transcription by 80% without reducing integration. To test whether transcription factors may recruit integrase to the insertion sites micrococcal hypersensitive sites within the promoter of *bub1-ade6* were mapped. The micrococcal sensitive sites corresponded well to the key positions of insertion, indicating that transcription factors may control the selection of target sites. To test this model further we studied the promoter of *fbp1* because its transcription factor binding sites are known. When *fbp1* was present in the target plasmid, 83% of the insertions occurred in the promoter. The majority of these were adjacent to the binding site of the Atf1/Pcr1 activator complex. A single nucleotide mutation known to block the binding of Atf1/Pcr1, blocked integration at the adjacent positions. This result supports the model that transcription factors recruit the integrase complex to target sites.

INDIVIDUAL MUTATIONS IN THE Q151M COMPLEX AFFECT AZT RESISTANCE AND POLYMERASE ACTIVITY

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The Q151M complex of mutations (A62V/V75I/F77L/F116Y/Q151M) in HIV-1 reverse transcriptase (RT) promotes resistance to many Nucleoside Reverse Transcriptase Inhibitors (NRTIs). Q151 interacts with the 3'-OH of an incoming dNTP and this interaction is partially disrupted if the incoming nucleoside triphosphate lacks the 3'-OH. Mutating Q151 would alter the interactions of the RT with dNTPs and NRTI-TPs. The Q151M mutation appears first, followed by the sequential appearance of the additional mutations, which appear to increase resistance to the NRTIs (Ueno et al). It has been suggested that the addition of the other mutations to Q151M may compensate for a loss in fitness caused by the Q151M mutation (Maeda et al). We have examined these mutations using *in vitro* assays.

In polymerase assays with normal levels of dNTPs (10-20 μM), Q151M by itself has polymerase activity similar to wild-type HIV-1 RT, while the Q151M complex has slightly decreased polymerase activity. When the concentration of dNTPs is sub-optimal (0.1-1.0 μM), Q151M has a slightly decreased ability to extend a labeled DNA primer while the Q151M complex is greatly affected. *In vitro* inhibition assays shows that Q151M is slightly less sensitive to AZTTP and is much less sensitive to ddNTPs compared to wild-type RT. Q151M and the Q151M complex also have a decreased ability to excise AZTMP from a blocked primer relative to wild-type HIV-1 RT. In our assays, the additional mutations in the Q151M complex greatly increase resistance to NRTI-TPs, in agreement with Ueno et al. However, the additional mutations do not appear to compensate for a loss in polymerase activity caused by Q151M; the Q151M complex appears to be a less fit RT than Q151M by itself. This suggests that increased resistance to NRTI-TPs caused by these mutations is the driving force for their selection rather than positive effect on polymerase activity.

NRTI therapy for HIV-2 infection can also select for the Q151M mutation; however, a Q151M complex does not appear to be selected. Q151M in the HIV-2 RT background has a slight effect on the ability of the RT to use dNTPs at sub-optimal levels, but overall the polymerase activity is similar to wild-type HIV-2 RT. The addition of Q151M to HIV-2 RT has a larger effect on resistance to NRTIs compared to the Q151M mutation in HIV-1 RT. This suggests that additional mutations are not needed because high-level resistance is obtained with a single mutation.

INTERACTIONS BETWEEN HIV-1 AND HIV-2: PROTEIN COMPLEMENTATION AND GENETIC RECOMBINATION

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HIV-1 and HIV-2 are genetically distinct viruses that individually can cause AIDS. Approximately one million people in the world are coinfected with HIV-1 and HIV-2. Additionally, these two viruses use the same (co)receptors and therefore can infect the same target cell populations. To explore the possible interactions between these two viruses, we examined whether the HIV-1 and HIV-2 Gag proteins can coassemble and complement each other, and whether these two viruses can recombine. Using two Gag mutants, we have demonstrated that HIV-1 and HIV-2 Gag proteins can complement each other's function to complete viral replication. Using bimolecular fluorescence complementation assay, we have also shown that these two Gag proteins can interact and coassemble into virus particles.

Viral RNA is specifically packaged into the virion via Gag-RNA interactions. The coassembly of HIV-1 and HIV-2 Gag proteins suggests that the RNAs from the two viruses can also be copackaged into the same virion, thereby allowing recombination to occur. To our knowledge, naturally occurring HIV-1/HIV-2 hybrid viruses have not been reported so far. It is unclear whether HIV-1 and HIV-2 can recombine, and if such recombination could occur, what would be the frequency of these events and the viability of the recombinants. To explore these questions, we examined whether modified HIV-1 and HIV-2 can recombine in a cell culture system. Each vector in our study contained a green fluorescence protein gene (*gfp*) with a different inactivating mutation. A functional *gfp* could be reconstituted via recombination, and generation of a functional *gfp* was used to score recombination events. We measured HIV-1 and HIV-2 recombination rates in four independent experiments and found that *gfp* was regenerated in approximately 0.2% of the infection events, which was 35-fold lower than the intrasubtype HIV-1 recombination rates. We isolated and characterized single GFP^+ cell clones and determined that all of them contained proviruses with reconstituted *gfp*. Using PCR primer sets specific to either HIV-1 or HIV-2, we mapped the general structures of the recombinant viruses, amplified portions of the recombinant viral genome by PCR, and characterized them by DNA sequencing. We observed several different recombination patterns and viruses with hybrid viral sequences.

Taken together, our study indicates that HIV-1 and HIV-2 can interact by protein complementation and genetic recombination. However, recombination occurs at low frequencies and multiple factors exist to restrict the generation of viable HIV-1 and HIV-2 hybrid viruses. Nevertheless, considering the large coinfected human population and the high viral load in patients, these rare events could provide the basis for the generation of novel human immunodeficiency viruses.

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VALIDATION OF THE TRANSLOCATIONAL EQUILIBRIUM OF HIV-1 RT AS A SPECIFIC TARGET

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A single cycle of nucleotide incorporation by the reverse transcriptase of the human immunodeficiency virus type 1 (HIV-1 RT), involves initial binding of the primer/template and an incoming nucleotide, a conformational change that traps the nucleotide, the formation of a new phosphodiester bond, the release of pyrophosphate (PPi), and ultimately polymerase translocation, which clears the nucleotide binding site. We have previously employed site-specific footprinting techniques to study the mechanism of HIV-1 RT translocation. Our data are consistent with a “Brownian ratchet” model of polymerase translocation, which postulates that the enzyme can rapidly oscillate between pre- and post-translocational states. The next complementary nucleotide acts like a pawl of a ratchet that traps the enzyme in the post-translocation state and prevents the reverse movement. Here we explored the concept of a ratchet model of HIV-1 RT translocation with regards to its implications for mechanisms involved in drug action and resistance associated with the pyrophosphate (PPi) analogue phosphonoformic acid (PFA or foscarnet). We identified “hot spots” for PFA-mediated inhibition of DNA synthesis during active elongation. Site-specific footprinting analyses revealed that the corresponding complexes exist predominantly in the pre-translocational state. The sensitivity to PFA is significantly reduced with sequences that show a bias toward the post-translocational state. These findings show that the translocational equilibrium is sequence dependent. It is rapidly established following the formation of each novel phosphodiester bond and the release of PPi, which helps to explain how the pre-translocated state can be accessed and trapped during active DNA synthesis. Thus, the bound inhibitor can be seen like a pawl of a ratchet and prevents the forward motion of HIV-1 RT. The proposed mechanisms of RT translocation and drug action are consistent with the PFA resistant phenotypes. We show that certain sequences and the PFA resistant E89K mutant diminishes the stability of the pre-translocated complex. In these cases, the enzyme is seen at multiple positions around the 3’ end of the primer. The results of this study validate the pre-translocated complex as a target for the development of novel, perhaps less toxic and more potent inhibitors that block HIV-1 RT translocation.

STRUCTURAL DETERMINATION OF AN RRE VARIANT RESISTANT TO *TRANS*-DOMINANT REVM10

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The nuclear export of both full length and partially spliced viral RNA in HIV-1 is highly dependent upon the interactions between the nuclear localization signal (NLS) of the viral Rev protein and a 234 nt viral RNA Rev Response Element (RRE). Disrupting this interaction has become an attractive target for antiretroviral drug design and gene therapy. In one therapeutic example, a *trans*-dominant negative Rev phenotype known as RevM10 (1,2) containing a mutation in the Rev nuclear export signal (NES) was genetically introduced (3,4) and thought to multimerize with wild type Rev thereby disrupting the viral RNA nuclear export process. However, Hamm et al. (5) demonstrated that two G to A mutations at nts 164 and 245 in the viral RRE RNA (RRE 61) allow the virus to escape the RevM10 therapy. This result suggests that the RRE also plays an important role in the process. Thus, our initial focus was to elucidate the structural role of the RRE in Rev binding. Using RNA SHAPE (6) chemistry, we first determined the *in-vitro* solution secondary structure for both the wild type RRE and RRE 61 RNA. Strikingly, a structural variation from wild type was observed in the III/IV/V region of the mutant RRE. We then probed the effects of the structural difference on Rev binding using various methodologies, including mass spectrometry. Our data suggests that Rev efficiently binds the stem I and IIb regions in both RNAs. Since the structural difference does not seem to affect Rev binding, we infer then that another unresolved cellular or viral factor must be involved that recognizes the RRE61 structural feature.

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HIV-1 REVERSE TRANSCRIPTASE DIMERIZATION AS AN ANTIVIRAL TARGET

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HIV-1 reverse transcriptase (RT) is a heterodimeric enzyme consisting of a 66kDa subunit (p66) and a p66-derived 51-kDa subunit (p51). Because RT's DNA polymerase activity is tightly coupled to the quaternary structure of the enzyme, HIV-1 RT dimerization is considered a target for antiviral drug development. In this regard, we have made significant progress in several key areas that are important for the identification and/or development of inhibitors of RT dimerization. These include: (i) the identification of "hot-spots" in the protein-protein interface that significantly contribute to the bulk of the RT inter-subunit binding energy; (ii) the development of high-throughput screening assays for HIV-1 RT dimerization; (iii) structure-activity relationships of TSAO-T derivatives, a unique class of compounds that destabilize the inter-subunit interactions of RT; and (iv) insight into how compounds that modulate RT dimerization might affect viral replication. This talk will review these key areas and critically assess RT dimerization as an antiviral drug target.

SELECTION OF MUTATIONS IN THE CONNECTION AND RNASE H DOMAINS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASE THAT INCREASE RESISTANCE TO 3'-AZIDO-3'-DIDEOXYTHYMIDINE

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Background: Recent work has suggested that mutations in the C-terminal domains of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) increases 3'-azido-3'-dideoxythymidine (AZT) resistance. Because it is not known if AZT selects mutations outside the polymerase domain of RT, we performed in vitro selections of HIV-1 with AZT and examined the entire coding sequence of RT for the emergence of resistance-related mutations.

Methods: Separate selections with AZT were carried out starting with wild-type or mutant virus encoding three AZT resistance mutations (M41L, L210W, T215Y). The viruses were passaged in MT-2 cells in increasing concentrations of AZT and AZT susceptibility (IC_{50}) was determined in P4/R5 cells that express beta-galactosidase under the control of the HIV-1 LTR. The entire coding region of RT from passaged viruses was RT-PCR amplified from HIV-1 RNA and the DNA was sequenced. Recombinant xxLAI virues, containing the mutational patterns selected in vitro, were generated by site-directed mutagenesis and were used to determine AZT susceptibility and NRTI cross-resistance using P4/R5 cells.

Results: The first resistance mutations to appear were 2 polymerase domain thymidine analog mutations (TAMs) – D67N and K70R – that together conferred 66-fold AZT resistance. These were followed by the acquisition of 2 novel mutations – A371V in the connection domain and Q509L in the RNase H domain – that in combination with D67N and K70R were associated with ~90-fold AZT resistance. Thereafter, the T215I mutation appeared but was later replaced by T215F, resulting in a large increase in AZT resistance (~16,000-fold). Site-directed mutants confirmed that A371V and Q509L together increased AZT resistance ~50-fold when in combination with TAMs. Mutagenesis studies also showed that HIV-1 containing D67N/K70R/T215F/A371V/Q509L conferred greater cross-resistance to lamivudine, abacavir and tenofovir than viruses without A371V/Q509L.

Conclusions: These results provide the first evidence that mutations in the connection and RNase H domains of RT are selected by AZT in combination with TAMs and confer significantly greater AZT resistance and cross-resistance to other nucleoside inhibitors. Biochemical studies are in progress to identify the molecular mechanism(s) involved.

POSTER ABSTRACTS

POSTER 1

DETAILED ANALYSES OF HIV-1 MATRIX MUTANTS: EFFECTS ON AN EARLY STAGE OF INFECTION

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We previously reported that the 20LK mutation in the HIV-1 matrix (MA) protein impairs the synthesis of HIV-1 viral DNA post-infection without disrupting virus assembly and release, RNA encapsidation, or Env incorporation into virions. We also extensively studied the effects of single amino acid changes throughout MA on virus particle assembly. Although many mutations showed defects in virus assembly and release, Gag processing, or Env incorporation into virions, the effects of these mutations on an early stage of the virus replication cycle were not examined.

In this study, we mainly sought to characterize the effects of three MA mutants (6VR, 49LD, 86CS) on early events in the virus replication cycle. The MA mutants showed modest or marked delay in their growth kinetics relative to WT (NL4-3) in various T-cell lines. Each of these three MA mutations does not largely affect virus production, RNA encapsidation, Env incorporation into virions, and Gag processing. Interestingly, we observe that the 6VR-induced defect is not reversed by pseudotyping with either the amphotropic murine leukemia virus envelope (ampho-MuLV Env) or VSV-G, whereas 49LD and 86CS are rescued by VSV-G but not ampho-MuLV Env. Preliminary data using immature VLP for 49LD suggest that interaction between gp41 and Gag in 49LD is stronger than that in WT, implying one possible cause of the defects. Real-time PCR analysis indicates that 6VR does not impair the viral DNA synthesis of early reverse transcription (RT) products, but reduces that of late RT products in an infected T-cell line. In contrast, 49LD and 86CS impair the viral DNA synthesis of both early and late RT products. These results suggest that the mutants 6VR, 49LD, and 86CS can be a useful tool to elucidate the role of MA in an early post-entry step. Ongoing experiments are underway to examine the effects of these MA mutations on virus-cell fusion and $tRNA^{lys3}$ incorporation into virions.

POSTER 2

THE L-SIGN CARBOHYDRATE RECOGNITION DOMAIN LIMITS HIV INTERACTIONS AND VIRUS TRANSMISSION

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Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) is a 44 kD C-type lectin that is expressed on myeloid-lineage DC, activated B cells, and some subsets of macrophages. DC-SIGN interacts with a wide variety of pathogens including HIV, SIV, CMV, Ebola virus, and dengue virus. Prior studies from our laboratory demonstrated that DC-SIGN-mediated HIV transmission is cell type dependent and could be recapitulated by human Raji B cells stably expressing DC-SIGN. In contrast to DC-SIGN, liver/lymph node-specific ICAM-3-grabbing nonintegrin (L-SIGN), a DC-SIGN-related molecule (also known as DC-SIGNR), is less effective in promoting the transmission of HIV when expressed in Raji B cells or other cell types. We sought to understand the molecular and functional differences between DC-SIGN and L-SIGN that underlie the difference in HIV transmission efficiency. Using a FACS-based binding assay with virus-like particles (VLPs) that incorporate HIV Env, we observed that VLPs interacted with DC-SIGN but not L-SIGN with 5, 6 and 7 repeats. To map the domains within DC-SIGN necessary for virus binding, eight Raji lines expressing DC-SIGN/L-SIGN-7 chimeras were created by overlap PCR. Wild-type and chimera lectin expression levels were measured using a cross-reactive monoclonal antibody. Examination of the chimeras revealed that replacement of the DC-SIGN CRD with the L-SIGN CRD was sufficient to impair VLP binding to background levels observed in Raji cell controls. By contrast, VLP binding was restored in L-SIGN chimeras containing the DC-SIGN CRD and further enhanced by inclusion of the DC-SIGN repeat domain. We next assayed HIV transmission by Raji cells expressing the different chimeric molecules. Consistent with the VLP binding data, we observed that the DC-SIGN CRD was both necessary and sufficient for HIV-1 transmission and the DC-SIGN repeat region could enhance the CRD transmission efficiency. Molecules containing the L-SIGN CRD did not support virus transmission. Finer mapping will be required to identify which amino acids within the DC-SIGN CRD are required for HIV capture and transmission. Despite its relatedness to DC-SIGN, our studies indicate that a direct contribution of L-SIGN to HIV replication in vivo may be limited.

POSTER 3

EXPLOITING RETROVIRAL PROTEIN CHEMISTRY FOR DIRECT FLUORESCENT LABELING OF VIRIONS TO STUDY HIV-1 AND SIV INTERACTION WITH DENDRITIC CELLS

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By exploiting the intrinsic chemistry of retroviruses, we have developed a novel method for generating fluorescently labeled virions with functional envelope glycoproteins. The method takes advantage of the fact that the internal proteins of retroviruses have cysteine residues present in thiol-form (S-H), while the surface proteins of retroviruses (inclulding the envelope glycoproteins SU and TM) have their cysteines present as disulfides (S-S). The free thiols of internal viral proteins thus present a potential target for fluorescent labeling with thiol-reactive reagents, without affecting the viral envelope glycoproteins. We used Alexa Fluor 488 maleimide to fluorescently label virions, and have biochemically characterized the resulting modifications. Alexa Fluor 488 labeling resulted in preferential covalent modification of key S-H-containing internal viral proteins, including nucleocapsid (NC), matrix (MA) and capsid (CA), while the envelope glycoproteins (SU, TM) remained unaffected. Dendritic cells (DCs) play an important role in AIDS pathogenesis, in part by facilitating the infection of T cells. Fluorescent virions are a useful reagent to study key aspects of virus/DC interaction, including binding, uptake, processing, and transmission of virions to T cells via “virological synapses”. Alexa 488-labeled HIV-1 and SIV virions were captured by immature and mature DCs in a dose-dependent manner. Alexa 488 labeled viruses exhibited characteristic intracellular localization at the cell periphery in immature DCs and in a deeper perinuclear location within mature DCs, results confirmed by parallel immunostaining for viral antigens. Alexa-labeled virions can be prepared with emission spectra in a variety of ranges and represent a useful reagent for monitoring DC-virus interplay and DC-associated virus spread. Contract No. NO1-CO-12400.

POSTER 4

MUTATION OF CA OVERCOMES A RATE-LIMITING BLOCK IN HIV-1 INFECTION OF MOUSE T CELLS

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We have previously described a postentry block to HIV-1 infection in mouse T cell lines that corresponded to a decrease in the nuclear accumulation of viral cDNA. In contrast, mouse fibroblasts (such as NIH3T3) of the same genetic background were permissive to HIV-1 replication after virus entry up to the point of integration. Here we demonstrate that mutations in HIV-1 CA can overcome the early block in mouse T cells and provide evidence that cellular factors regulate this step of replication. We have recently identified an antiviral protein, C-terminally truncated CPSF6, that prevents the nuclear localization of functional HIV-1 pre-integration complexes (PICs). Selection for HIV-1 resistant to truncated CPSF6 identified residues in CA that regulate this interaction. Because the CA mutant viruses could overcome an apparent nuclear entry restriction imposed by truncated CPSF6, we tested whether they could overcome a similar block in the mouse TA3 thymoma cell line. Notably, the three tested mutant HIV-1 isolates were all more efficient in the infection of mouse TA3 cells. In particular, HIV-1 with the CA N74D mutation restored the infection efficiency in mouse TA3 cells to levels observed in NIH3T3 cells. The infection kinetics of wild-type and N74D virus in TA3, Hut78, and NIH3T3 cells also revealed that wild-type HIV-1 was rate-limited at high titers in mouse TA3 cells and to a lesser extent in Hut78 cells, suggesting that necessary cellular factors were limiting in the lymphocytic lines. In support of this observation, fusion of TA3 cells with human 293T cells rescued the infection defect. Finally, introduction of truncated CPSF6 in the TA3, Hut78, and NIH3T3 cells yields different levels of antiviral activity. The least antiviral activity was detected in the TA3 cells and greatest level was detected in the NIH3T3 cells, suggesting that the rate-limiting factors are the same that truncated CPSF6 targets. Collectively, these data suggest that cell factors necessary for nuclear entry of HIV-1 are diminished in mouse T cells and that HIV-1 CA interacts with these factors to regulate PIC nuclear entry. Our data also indicate that HIV-1 with CA N74D has desirable properties as a vector as it can establish higher multiplicity infection in both mouse and human T cell lines relative to wild-type HIV-1.

*these authors have made comparable contributions

POSTER 5

THE MOUSE LEM DOMAIN PROTEINS EMERIN AND LAP2α ARE DISPENSABLE FOR HIV-1 AND MLV INFECTION

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Productive retroviral infections lead to integration of the viral genome into the host cell genome. The human immunodeficiency virus 1 (HIV-1) can infect both dividing and non-dividing cells, while the murine leukemia virus (MLV) infects only dividing cells. Following reverse transcription, the viral pre-integration complexes (PICs) must mediate integration into the genome. Two LEM domain containing nuclear membrane proteins, emerin and the lamina-associated polypeptide (LAP) 2α, that are known to interact with Barrier-to-autointegration factor (BAF) have been suggested to be essential for productive HIV-1 and MLV infection and are believed to mediate association of viral PICs with the host cell chromatin. In this study, we examined whether HIV-1 or MLV could infect primary cells from mice deficient for emerin, Lap2α or both emerin and Lap2α. HIV-1 and MLV infectivity in mouse embryonic fibroblasts (MEFs) from emerin knock-out, Lap2α knock-out or emerin and Lap2α double knock-out mice was comparable to infectivity in wild-type littermate-derived MEFs, indicating that both emerin and Lap2α were dispensable for HIV-1 and MLV infection of dividing, primary mouse cells. Moreover, because emerin has been suggested to be important for infection of human macrophages by HIV-1, we also examined HIV-1 infectivity in macrophages from wild-type mice or mice deficient in emerin, Lap2α or both emerin and Lap2α. Since the presence or absence of either emerin or Lap2α does not alter HIV-1 infectivity in the mouse macrophages, we conclude that these two LEM domain-containing proteins are not required for infection of non-dividing, mouse cells by HIV-1.

POSTER 6

DISRUPTION OF POSTENTRY HIV-1 REPLICATION IN DIVIDING AND NONDIVIDING CELLS BY MUTANT CPSF6

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The synthesis of viral cDNA (vDNA) from genomic RNA and the insertion of the linear vDNA into the host cell chromatin are defining characteristics of retroviral replication. While the role of virion proteins in the postpenetration retroviral replication has been the subject of intense scrutiny, the participation of host cell factors in these early events is poorly understood. Here we present evidence that a C-terminally truncated form of the pre-mRNA 3'-end processing factor CF Im-68 (also known as CPSF6) specifically interferes with the postentry replication of HIV-1. C-terminally truncated CPSF6 causes functional HIV-1 pre-integration complexes (PICs) to be trapped in the cell cytoplasm. Passage of HIV-1 in dividing cells expressing truncated CPSF6 selects for changes in CA and alters HIV-1's ability to infect nondividing cells. While certain CPSF6-resistant viruses are impaired in the infection of growth arrested HeLa cells, the relative susceptibility to truncated CPSF6 also predicts the efficiency of HIV-1 infection of terminally differentiated macrophages. In summary, our findings identify HIV-1 CA determinants necessary for the infection of nondividing cells and suggest that key cell factors that regulate HIV-1 nuclear entry are targeted by truncated CPSF6.

*these authors have made comparable contributions

POSTER 7

SELECTION FOR HIV-1 RESISTANT CELLS BY RNA INTERFERENCE

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Host genes profoundly affect the susceptibility of mammalian cells to infection by retroviruses. However the identity of host cell factors that regulate HIV replication postpenetration to the point of integration has remained elusive. In an attempt to identify positive cofactors in early stages of HIV replication, we transduced HeLa cells with an FIV vector encoding an shRNA library (8,500 target genes; 45,000 unique shRNAs). After extensive screening with HIV expressing an eGFP marker gene (HIV-eGFP), shRNA-positive, HIV-eGFP-resistant HeLa cell clones were identified. Isolated cell clones displayed were at least 10-fold resistant to HIV-eGFP using amphotropic murine leukemia virus vector (A-MLV) Env or VSV-G entry pathways. By contrast, infection by MLV or FIV vectors using the same entry pathways was comparable or slightly diminished relative to parental HeLa cells. These data suggested a postentry block, specific to HIV in the cell clones. Experiments with HIV-SVluc and HIV-CMVRFP vectors that express marker genes independent of the HIV-1 LTR yielded similar results and suggest that the infection block may be prior to integration. At least 8 unique shRNA sequences have currently been identified in different restrictive cells and are being examined for antiviral function. In addition, a novel mutation in Tat was identified in the screen in a subset of cells harboring latent HIV-1 proviruses. Evaluation of the Tat latency mutation and validation of shRNA targets for HIV-1 restriction will be presented.

POSTER 8

BIOINFORMATIC AND BIOCHEMICAL ANALYSIS OF PROTEIN-PROTEIN INTERFACES IN POLIOVIRUS POLYMERASE LATTICES

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The poliovirus replication complex involves the viral RNA-dependent RNA polymerase in oligomeric complexes on the surface of membranes. The poliovirus polymerase assembles into two-dimensional lattices that display cooperative catalytic activity (Science 296:5576, 2002) and viruses that contain polymerase mutations have been shown to be dominant negative(Nat. Genet 37:701, 2005). Lattice formation requires two sets of polymerase-polymerase contacts; characterizing this protein-protein interaction is an important step in the design of novel antiviral therapies. One polymerase-polymerase interaction site, Interface I, has been identified and validated by previous work (Structure 5:1109 1997; EMBO 20:1153 2001, JBC 277:31551 2002). Interface I is an asymmetric interaction which forms extendable head to tail fibers of polymerase.To form planar lattices, a second oligomeric interface must exist to stack up interface I fibers. To generate plausible hypotheses about this second oligomeric interface, computational modeling was employed.

To model the potentially alternative polymerase conformations that might be sampled, low frequency harmonic oscillations were calculated using normal mode analysis. Ten such alternative conformations were modeled into polymerase-polymerase fibers. The surface convolution was calculated for one hundred pairs of fibers, creating thousands of complexes. The complexes were then filtered for those that formed interactions that could be propagated into large lattices. Selection of the symmetric and parsimonious interfaces within the family of parallel and anti-parallel sheets resulted in four candidate interfaces involving distinct patches of residues on the polymerase. Multiple sequence alignment of every known picornavirus polymerase exhibits high conservation and co-variation within the regions that make up the postulated second interface. The wavelength-dependence of turbidity during protein oligomerization will be used to evaluate the kinetics and extent of polymerase oligomerization for wild type polymerase and polymerase that contains mutations designed to disrupt the posited second interface.

POSTER 9

THE NUCLEOSIDE RESISTANCE MUTATIONS L74V AND M41L+T215Y CAN EACH COMPENSATE FOR THE REDUCTION IN REPLICATION FITNESS CONFERRED BY THE NON-NUCLEOSIDE (NNRTI) RESISTANCE MUTATIONS K101E+G190S

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We showed that the combination of the NNRTI resistance mutations K101E+G190S markedly reduces the replication fitness of HIV-1_{NL4-3}, using growth competition assays in PM1 cells. We screened clinical RT sequences, in order to identify second-site mutations that might compensate for the reduced fitness of K101E+G190S. We identified a clinical RT clone, D10, which contains the nRTI resistance mutations M41L+T215Y in combination with K101E+G190S, in addition to several polymorphisms. We produced a chimeric NL4-3(D10 RT) virus from pNL4-3 that had the D10 RT sequence substituted for NL4-3 RT. NL4-3(D10 RT) showed markedly improved fitness compared to NL4-3(K101E+G190S). In order to understand whether the nRTI resistance mutations in D10 accounted for the improved replication fitness, we performed growth competition assays in PM1 cells between two site-directed mutants of NL4-3, K101E+G190S and M41L+T215Y+K101E+G190S. We found that the nRTI+NNRTI-resistant quadruple mutant had markedly improved fitness in vitro compared to the NNRTI-resistant double mutant. Because L74V has been reported to improve the replication fitness of the NNRTI-resistant mutants G190E and L100I+K103N, we also constructed and tested the L74V+K101E+G190S NL4-3 mutant against K101E+G190S alone. We found that L74V also improved the replication fitness of K101E+G190S. These studies demonstrate that nRTI resistance mutations can compensate for the reductions in replication fitness observed with some NNRTI resistance mutations, and that these effects are not limited to L74V. These findings indicate that interactions between other nRTI and NNRTI resistance mutations should be investigated. Since replication fitness appears to influence the frequency of resistance mutations occurring in patients, these studies suggest that giving different nRTIs in combination with an NNRTI may influence the genetic basis for NNRTI resistance during treatment failure.

POSTER 10

REPLICATION DEFECT OF K65R MUTANT OF HIV-1 RT IS PARTIALLY COMPENSATED BY ADDITION OF THE A62V AND S68G MUTATIONS

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Background: The K65R mutation in HIV-1 reverse transcriptase (HIV-1 RT) reduces drug susceptibility to abacavir, didanosine, tenofovir, and stavudine. Despite wide use of these drugs K65R remains relatively rare, possibly due to its impaired replication capacity. When it does occur, K65R is frequently associated with the A62V and/or S68G mutations (20% and 40% of sequences, respectively). In the current study we hypothesized that A62V and S68G mutations provide a replication advantage for the HIV-1 viruses containing K65R mutation.

Methods: Viruses containing the K65R HIV-1 RT mutation alone or in combination with A62V and S68G mutations were created by site-directed mutagenesis. To monitor viral replication in a multi-cycle competition assay, we created two LAI- based vectors by introducing two silent mutations at polymorphic sites located in close proximity to the K65R codon to avoid genetic recombination. Mutant viruses were subcloned into a pair of LAI-based vectors with markers which were detected by allele-specific real-time RT-PCR (MultiCode RTx, EraGen Biosciences). Relative fitness was calculated by using the equation $(s+1) = \exp \{1/(t_2-t_1)\ln\{[N_1(t_2)/N_2(t_2)][N_2(t_1)/N_1(t_1)]\}$, where $t_{1 \text{ or } 2}$ are time points;N_1 and N_2 are the numbers of respective virions at a particular time point.

Results: Control competition experiments demonstrated that the silent marker mutations did not affect replication fitness. In this test system, the K65R mutation was shown to strongly impair viral replication in comparison to the WT virus. Results of direct competition between K65R mutant virus and K65R+A62V, K65R+S68G, or K65R+A62V+S68G. indicated that either double-mutant was more fit than the K65R alone and the triple-mutant had increased fitness relative to the double mutants. To asses the replication capacity of the triple-mutant we performed competition experiments between K65R+A62V+S68G and WT viruses. While addition of the A62V/S68G mutations increased fitness of the K65R mutant, the triple mutant K65R+A62V+S68G still showed replication defect compared to WT. Quantification of the relative fitness indicated that replication capacity of the viruses were in the following order: K65R < K65R+A62V < K65R+S68G < K65R+A62V+S68G < WT.

Conclusions: Our findings support the hypothesis that A62V and S68G act as compensatory mutations for K65R by partially restoring the viral fitness.

POSTER 11

MECHANISMS THAT PREVENT TEMPLATE INACTIVATION BY RNASE H CLEAVAGES THAT OCCUR DURING REVERSE TRANSCRIPTION

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The RNase H activity of HIV reverse transcriptase (RT) cleaves the RNA template concomitant with DNA synthesis and is required for viral replication. In our previous study, we examined template cleavages at the base of a hairpin structure generated during synthesis on the EIAV template and showed that they were likely created from a 3'-end directed primary and secondary cleavage mechanism mediated by RT while paused during synthesis. In this study we report that all the prominent cleavage fragments generated during primer extension correlated with cuts derived from pause sites on this template. Of these, the cleavage fragments that persisted throughout the reaction correlated with 3'-end directed secondary cuts. The mechanism by which these secondary cuts were generated created a template fragment with little overlap with the 3' terminus of the primer. Therefore, we investigated whether these cuts could lead to dissociation of the primer from the template. Using a model template system we found that generation of 3'-end directed secondary cuts inactivated the primer for further extension. This observation provided a plausible mechanism to explain the persistence of cleavage products during synthesis on the EIAV template. Moreover, this result suggested that 3'-end directed secondary cleavages could be detrimental to viral replication by inactivating primer extension.

Addition of nucleocapsid protein (NC), the virally encoded nucleic acid chaperone protein, reduced pausing and the generation of pause-related secondary cuts on the EIAV template. This suggested a role for NC in preventing the RNase H cleavages that could be detrimental to viral replication. Likewise, improving the efficiency of synthesis by increasing the concentration of dNTPs produced similar results: reduced pausing and pause-related secondary cuts. Finally, we considered another situation in which RNase H-induced template inactivation would take place: the halting of synthesis by incorporation of a nucleoside analogue. We report that RT generated 3'-end directed primary and secondary cleavages on an AZT-terminated substrate even under conditions that allowed considerable RT-mediated AZT excision and subsequent primer extension. Overall, the results identify a means by which HIV may facilitate primer extension so that synthesis-associated RNase H cleavage does not inactivate the viral genome.

POSTER 12

3'-AZIDO,3'-DEOXYTHYMIDINE-(5')-TETRAPHOSPHO-(5')-ADENOSINE, THE PRODUCT OF ATP-MEDIATED EXCISION OF CHAIN-TERMINATING AZTMP, IS A POTENT CHAIN-TERMINATING SUBSTRATE FOR HIV-1 REVERSE TRANSCRIPTASE

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HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT) involves phosphorolytic excision of chain-terminating AZT-5'-monophosphate (AZTMP). Both pyrophosphate (PPi) and ATP act as excision substrates *in vitro*, but the intracellular substrate used during replication of AZT-resistant HIV is still unknown. PPi-mediated excision produces AZT-5'-triphosphate (AZTTP), which could be immediately re-used as substrate for viral DNA chain-termination. In contrast, ATP-mediated excision produces the novel compound AZT-(5')-tetraphospho-(5')-adenosine (AZTp4A). Since little is known of the interaction of AZTp4A with HIV-1 RT, we carried out kinetic and molecular modeling studies to probe this. AZTp4A was found to be a potent inhibitor of HIV-1 RT catalyzed DNA synthesis and of both ATP- and PPi-mediated AZTMP excision. AZTp4A is in fact an excellent chain-terminating substrate for AZT-resistant RT-catalyzed DNA synthesis, better than AZTTP (k_{pol}/K_d = 6.2 and 11.9 for AZTTP and AZTp4A, respectively). The affinity of AZT resistant HIV-1 RT for AZTp4A is at least 30000-fold greater than that for the excision substrate ATP, and about 10-fold greater than that for AZTTP. Dissociation of newly formed AZTp4A from RT may therefore provide a significant rate-limiting step for continued HIV-1 DNA synthesis. Our studies show that the products of PPi- and ATP-mediated excision of chain-terminating AZTMP (AZTTP and AZTp4A, respectively) are both potent chain-terminating substrates for HIV-1 RT, suggesting that there is no obvious benefit to HIV using ATP instead of PPi as excision substrate.

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POSTER 13

EFFECT OF NUCLEOTIDE SUGAR STRUCTURE ON RECOGNITION AND USE BY HIV-1 REVERSE TRANSCRIPTASE

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Nucleoside reverse transcriptase inhibitors (NRTI) are an important class of therapeutics used to treat HIV-1 infection. Unfortunately, prolonged NRTI therapy invariably results in viral resistance to the drugs. A major mechanism for NRTI resistance is phosphorolytic excision of the incorporated NRTI. While this mechanism predominates in resistance to AZT, phosphorolytic excision may play some role in resistance to most if not all therapeutic NRTI. Structurally modified nucleotides that can be readily incorporated into viral DNA, but once incorporated are poorly extended and/or refractory to excision may be of potential use in the treatment of NRTI-resistant HIV-1. In the present study, we used a combination of pre-steady state and steady state kinetics to investigate the role of nucleotide sugar structure on the incorporation, extension and excision activities of HIV-1 RT. RT-catalyzed incorporation rates were 2'-deoxycytidine-5'-triphosphate (primarily C2'-endo) > aracytidine-5'-triphosphate (O4'-endo) >> cytidine-5'-triphosphate (primarily C3'-endo). Once the nucleotides were incorporated, the order of RT-catalyzed extension from the 3'-terminal cytidine nucleotide was 2'-deoxycytidine-5'-triphosphate > cytidine-5'-triphosphate >> aracytidine-5'-triphosphate. Inclusion of ara-CTP in RT polymerization reactions resulted in significant and prolonged polymerization pausing at the point of ara-CMP incorporation. RT-catalyzed phosphorolytic excision rates were dCMP > araCMP > CMP; the results were the same whether ATP or pyrophosphate was used as excision substrate. Our data suggest that RT-catalyzed nucleotide incorporation, nucleotide extension, and nucleotide excision, all respond differently to different nucleotide sugar conformations. The facile incorporation but difficult extension and excision of nucleotides with arabinose sugar structures suggest that such sugars may be useful in the development of new NRTI active against drug resistant HIV-1 variants.

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POSTER 14

EXAMINING INTERACTIONS OF HIV-1 REVERSE TRANSCRIPTASE WITH SINGLE-STRANDED TEMPLATE NUCLEOTIDES BY NUCLEOTIDE ANALOG INTERFERENCE

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The structures of nucleic acid-containing human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) have been instrumental in dissecting the multi-functional properties of this key retroviral enzyme for antiviral therapy. In particular, co-crystals of RT with duplex DNA and an RNA/DNA hybrid have highlighted critical contacts mediated by protein motifs of its N-terminal DNA polymerase and C-terminal RNase H domains. These studies have implicated several residues of the p66 fingers subdomain of HIV-1 RT in contacting the single-stranded template overhang immediately ahead of the DNA polymerase catalytic center. This interaction presumably assists to induce the appropriate geometry on the templating base for efficient and accurate incorporation of the incoming dNTP. While a number of studies have investigated the geometry of duplex nucleic acid positioned between the catalytic centers of HIV-1 RT, the manner in which the single-stranded template traverses the p66 fingers subdomain prior to accessing the DNA polymerase catalytic center is less well understood. To investigate this, we have introduced nucleoside analogs, either individually or in tandem, into the DNA template ahead of the catalytic center and investigated whether they induce pausing of the replication machinery prior to serving as the templating base. Analogs included abasic linkages, neutralizing methylphosphonate linkages and conformationally-locked nucleosides. Several Phe61 mutants were included in our analysis, based on previous data indicating that altering this residue affects both strand displacement synthesis and the fidelity of DNA synthesis. Our results demonstrate that altering the topology of the template strand two nucleotides ahead of the catalytic center can interrupt translocation. Mutating Phe61 to either Ala or Leu accentuates the translocation defect, while replacement with an aromatic residue (Trp) allows the mutant enzyme to bypass the template analogs with relative ease. Collectively, our data indicate that altering template geometry 1-2 nt ahead of the catalytic center influences protein:nucleic acid contacts to stall HIV-1 RT and this effect can be modulated by Phe61 substitutions. Although crystallographic data depicted important interactions between the fingers subdomain of HIV-1 RT and the single stranded template, their significance during the dNTP catalysis is unclear. Our nucleoside analog strategy sheds light on the importance of these interactions.

POSTER 15

THE IDENTITY OF DIMERIZATION INITIATION SIGNAL AFFECTS THE FREQUENCIES AND DISTRIBUTIONS OF INTERSUBTYPE RECOMBINATION JUNCTIONS

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* Equal contribution

Frequent recombination occurs during HIV-1 reverse transcription; genetically different recombinants can be generated from virions containing two different RNAs (heterozygous virions). We previously demonstrated that HIV-1 subtypes B and C can recombine, albeit at a rate lower than intrasubtype recombination, by using a subtype B- and a subtype C-based vector, each containing a mutant green fluorescence protein gene (*gfp*), and measured the frequency of *gfp* reconstitution. To identify crossover junctions between the viral genomes of subtypes B and C, we isolated 56 GFP^+ cell clones containing recombinant proviruses and sequenced a 5-kb region of the viral genome comprising the 5' LTR, 5' untranslated region, *gag*, and *pol*. A total of 56 crossover junctions were identified from these 56 proviruses. We observed a recombination gradient – there are significantly more recombination events at the 3′ half of the 5-kb than the 5′ half ($p = 0.002$).

HIV-1 subtypes B and C have different sequences in the dimerization initiation signals (DIS). We hypothesized that this DIS difference affected proper dimer formation at the 5′ end of the RNA, thereby led to the uneven distribution of the crossover junctions. To test our hypothesis, we examined the recombinants generated from a subtype C virus and a modified subtype B virus containing a 2-nt change in the DIS to match that of the subtype C virus (B-Cdis). We isolated 56 GFP^+ cell clones generated from B-Cdis and C viruses and sequenced the same 5-kb viral genomes. We identified 96 crossover junctions in 56 recombinants, which is significantly higher than the B/C recombinants ($p = 0.003$). Furthermore, the crossover junctions were distributed more evenly throughout the 5-kb region.

In summary, our results indicate that mismatches in the DIS sequences not only affect RNA dimerization and packaging but also provides an important function during the reverse transcription of the viral RNA. Therefore, these results also provide insights into the structure of the viral RNA during reverse transcription.

POSTER 16

FIDELITY OF HIV-1 REVERSE TRANSCRIPTION

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Due to the low fidelity of HIV-1 reverse transcriptase (RT), viral replication results in the generation of extensive genetic diversity. This diversity makes it possible for the virus to evade host immune responses and generate viral variants that are resistant to antiretroviral therapies. We developed a yeast-based assay (TyHRT) for HIV-1 RT activity to look for novel inhibitors, characterize RT variants and monitor the evolution of RT drug resistance in HIV-1 infected individuals. The TyHRT assay has been used previously to establish the frequency of resistance in NNRTI-naïve populations as both an indicator of predicted efficacy for NNRTIs and as a measure of the genetic diversity that exists during active infection. The frequency of drug resistance varies among individuals suggesting that discrete viral populations have different levels of genetic diversity. These differences in diversity may reflect the altered replication fidelity of RT variants present in the populations.

In order to test the fidelity HIV-1 RT variants, we furthered developed an assay to test mis-incorporation and slippage during reverse transcription. A series of fidelity reporter genes were placed in TyHRT elements such that RT-mediated mis-incorporation and slippage result in the reversion of mutant *trp1* alleles. This assay was used to isolate RT variants with altered levels of fidelity from clinical samples. This assay is also being used to screen a mutagenized library for additional RT fidelity variants.

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POSTER 17

ACETYLATED-INHIBITORS OF HIV-1 INTEGRASE

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Using affinity acetylation and mass spectrometry we previously identified that a small molecule acetylated-inhibitor MDACS (methyl N,O-bis(3,4-diacetoxycinnamoyl)serinate) preferentially binds at the HIV-1 integrase (IN) dimer interface. Our efforts have been extended in the present study to better understand the mechanism of acetylation and determine binding modes of related small molecule inhibitors. Comparison of di-acetyl and mono-acetyl derivatives indicated significantly enhanced reactivity for the former compounds. A double bond in conjugation between the aryl ring and the ester carboxylate significantly facilitated acetylation. Based on these results a hypothetical acetylation mechanism is proposed. We then compared several structurally related compounds to dissect features that aid MDACS in its binding to the architecturally and functionally important IN dimer region. A striking difference was observed between MDACS and acetylated chicoric acid in their interaction with the dimer interface. The only structural difference between MDACS and acetylated-chicoric acid is that the former contains a methyl ester group within its linker region, while the latter possesses two carboxylic acids in its linker segment. Application of di-acetyl functionality was extended to examine binding of an acetylated-CAPE (caffeic acid phenyl ethyl ester) to HIV-1 integrase. Our data indicate that acetylated-CAPE binds tightly at multiple sites on the protein including the amino acids essential for IN catalytic activity. Dissecting structural foundations of how inhibitors interact with the protein is important for better understanding of HIV-1 IN structure as a therapeutic target and rational development of new antiviral compounds.

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POSTER 18

SURFACE TOPOLOGY ANALYSIS OF FULL LENGTH HIV-1 INTEGRASE

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HIV-1 integrase (IN) consists of three distinct structural and functional domains. The N-terminal domain (residues 1-50) contains conserved pairs of histidine and cysteine residues that bind zinc. This domain is thought to serve an architectural role. The core domain (residues 51-212) comprises the catalytic site. Three acidic residues, D64, D116 and E152 play a key role in coordinating catalytic divalent metal ions. The C-terminal domain (residues 213-270) is thought to nonspecifically bind the DNA substrate. Recently two multi-domain crystal structures of HIV-1 IN have emerged comprising either the core and C-terminal domains, or N-terminal and the core domains. However, tremendous efforts to refine the full-length HIV-1 IN structure with crystallography or NMR have been impeded by the low solubility of the protein. In the present work, we employed an alternative approach that allows to probe surface topology of the protein using small molecule group specific modifiers followed by mass spectrometric analysis. In particular, we used lysine, arginine and tryptophan specific reagents NHS-biotin, hydroxyphenylglyoxal and dimethyl(2-hydroxy-5-nitro-benzyl)sulfonium bromide, respectively. In these experiments the two truncated protein constructs: N-terminus plus core domain and core domain plus C-terminus, for which X-ray structural data are available, were compared with a full length IN. Our experimental results together with the available structural data on the truncated proteins enable to generate a plausible molecular model for the full length IN.

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POSTER 19

INHIBITION OF HIV-1 CONCERTED INTEGRATION BY STRAND TRANSFER INHIBITORS WHICH RECOGNIZE A TRANSIENT STRUCTURAL INTERMEDIATE

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Human immunodeficiency virus type-1 integrase (IN) inserts the viral DNA genome into the host chromosomes. The diketo acid (DKA) inhibitors and their derivatives effectively inhibit HIV-1 replication by preventing integration in vivo. The strand transfer inhibitors L-870,810, L-870,812 and L-841,411, at low nM concentrations, effectively and selectivity inhibited the concerted integration of viral DNA substrates in vitro. The naphthyridine carboxamide inhibitors L-870,810 and L-870,812 significantly modified the structure of the strand transfer complex (STC) formed with a blunt-ended substrate and circular DNA target, as determined by native agarose gel electrophoresis. The structurally modified STC lacks strand transfer capabilities. The modified STC produced in the presence of L-870,810 appears related to an early form of the STC, identified as a transient precursor to the STC. The precursor STC and modified STC consists of only free donor non-covalently bound by IN to target, while the STC contains predominantly the concerted product. The structure of the STC formed with a substrate containing a 3' OH recessed end was not modified by these inhibitors. In contrast to the blunt-ended substrate, the 3' OH recessed substrate required significantly higher concentrations of inhibitor to achieve effective inhibition of concerted integration and other strand transfer products. We determined that a presumed transient intermediate is required for effective inhibition of concerted integration. The results suggest that the IN-viral DNA complex is "trapped" by the strand transfer inhibitors within the PIC prior to nuclear transport.

POSTER 20

MECHANISM OF HUMAN APOBEC3G-MEDIATED RESTRICTION OF THE YEAST ENDOGENOUS RETROELEMENT TY1

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Several mammalian APOBEC3 proteins and most prominently human APOBEC3G have been shown to inhibit the replication of a variety of different retroviruses and retrotransposons. APOBEC3G-dependent deamination of retroelement cDNA cytosines is a clear hallmark of this innate restriction system, however, a deamination-independent mechanism has also been documented. Here, heterologous expression of human APOBEC3G in yeast is used to evaluate the contribution of deamination-dependent and -independent mechanisms to the restriction of the retrotransposon Ty1. Restriction does not require the major uracil excision enzyme of yeast, the Ung1 protein. Moreover, like some (but not all) HIV-1 reports, mutational analyses indicate that each of the conserved zinc-binding domains of APOBEC3G can alone mediate restriction. Thus, the results combine to suggest a conserved retroelement restriction mechanism in which human APOBEC3G can elicit both deaminase-dependent and -independent modes of inhibition.

POSTER 21

THE BIOCHEMICAL PARAMETERS OF APOBEC3G ENZYMATIC ACTIVITY AND ITS INHIBITION BY HIV-1 VIF

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Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is a member of a cellular cytidine deaminase family involved in diverse cellular processes including cell metabolism, immunoediting, endogenous retrotransposition and viral infection. APOBEC3G incorporated into viral particles in virus producing cells restricts retroviral infection by hypermutating cytidine residues on the newly synthesized viral cDNA minus strand during reverse transcription in target cells. APOBEC3G mediated deamination impedes reverse transcription and leads to multiple G→A substitutions on the plus strand, resulting in the production of non-infectious particles. HIV-1 Vif allows productive viral replication in HIV-1 natural target cells by mediating the ubiquitination of APOBEC3G and its subsequent degradation in the proteasome, reducing the packaging of APOBEC3G into the progeny virions. Using a novel cell-free system for measuring the deaminase activity of purified, human-cells derived APOBEC3G, we have performed a comprehensive biochemical analysis on the kinetics of APOBEC3G enzymatic activity and found that A3G has a Km of approximately $7.4*10^{-9}$M for a synthetic ss-deoxyoligonucleotide. Addressing the possibility that Vif is capable of attenuating this activity, we have found that the purified HIV-1 Vif protein efficiently inhibits the enzymatic activity of APOBEC3G in vitro. We mapped the regions involved in APOBEC3G inhibition using a battery of Vif derived peptides. Two regions are suggested to exert the observed inhibition, corresponding to Vif (25-39aa) and Vif (105-119aa). These results imply that Vif might play a role in the inhibition of APOBEC3G deaminase activity inside the virion or at the early phase post-entry to the target cells, suggesting a multifaceted counter-APOBEC3G mechanism employed by Vif. Understanding the nature of this interaction between Vif and APOBEC3G could lay the basis for the development of novel anti-Vif compounds.

POSTER 22

HIV STRUCTURAL DATABASE: A STRUCTURAL RESOURCE FOR INDUSTRIAL AND ACADEMIC RESEARCHERS TO FACILITATE RATIONAL DRUG DESIGN

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Federal agencies, academia, and industry have invested heavily in the development of structural protein and chemical databases. A hurdle to realizing the benefits of this information is that the data are distributed over several public and private archives, leading to issues of interoperability. Technical challenges relating to the integration of data weaken the appeal of bioinformatics databases. The issue, however, creates an ongoing struggle in the broader life science research setting and has to be resolved in order to provide maximum possible value and eliminate incompatibilities. To maximize the return on this investment, the information must be widely accessible by a broad community of scientific researchers. NIST, in collaboration with NCI, NIAID, and Rutgers University has been working on the development of an integrated 2-D and 3-D structural data resource for AIDS (http://xpdb.nist.gov/hivsdb/hivsdb.html). HIV-Structural-DB has obtained structural data and related information for HIV proteins from various sources and compiled these data for efficient retrieval. A novel technique combining chemical taxonomy, data-tree, and Chem-BLAST is used to analyze, annotate, integrate, and set customized queries to extract both 2-D and 3-D structural information, empowering the research community to more effectively understand and use structural information towards HIV research. The data annotation and distribution techniques used in this database lays the foundation for semantic web technology for biological data with special emphasis to rational drug design by the mix and match approach. The data, annotation and distribution techniques will be presented. The HIV-Structural-DB was recently extended to include drug resistance information about drugs approved by the FDA and mutants that are associated with the drugs. An informative gallery (http://xpdb.nist.gov/hivsdb/gallery.html) was created to show pictures of FDA-approved drugs, drug-resistant mutants, and mutation animations. In addition, it provides mutated drug-resistant structures for download and links to citations.

POSTER 23

IN VITRO VIRAL RESISTANCE TO PA-457, A NOVEL INHIBITOR OF HIV-1 MATURATION

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Background: The identification of new anti-HIV-1 drugs targeting novel sites of action remains a high priority in this era of drug-resistant viruses. One such new drug, PA-457, potently inhibits HIV-1 by blocking a late step in the Gag processing pathway, specifically the cleavage of SP1 from the C-terminus of capsid (CA).

Methods: To gain insights into the mechanism(s) by which HIV-1 could evolve resistance to PA-457, we sought to identify and characterize the full spectrum of HIV-1 variants capable of conferring resistance to this compound. PA-457-resistant viral isolates were selected *in vitro* by serial passage at a suboptimal PA-457 concentration.

Results: Numerous independent rounds of selection repeatedly identified six single amino acid changes that independently confer PA-457 resistance: three at or near the C-terminus of CA (H226Y, L231F and L213M) and three at the 1st and 3rd residues of SP1 (A1V, A3T and A3V). PA-457-resistance was demonstrated by restoration of CA-SP1 cleavage in the presence of PA-457. Virus replication kinetics demonstrated that the mutations in CA and SP1-A1V do not impose a significant replication defect in culture. In contrast, mutations A3V and A3T severely impaired virus replication and inhibited core condensation. The replication defect imposed by A3V was reversed by a second-site compensatory mutation in CA (G225S). Intriguingly, high concentrations of PA-457 enhance replication capacity and maturation of the SP1 residue 3 mutants. The PA-457-resistance-conferring mutations are currently being characterized in the context of a PI-resistant protease.

Conclusions: The clustering of mutations conferring resistance to PA-457 at the CA/SP1 junction confirms that this region is the major target of PA-457 activity. The different phenotypes associated with resistance-conferring mutations suggest the existence of multiple mechanisms by which HIV-1 can evolve resistance to PA-457. Interestingly, all amino acid positions to which PA-457-resistance maps *in vitro* are highly conserved among HIV-1 isolates, suggesting that there may be a fitness cost to the development of PA-457 resistance *in vivo*, a pertinent matter as PA-457 (or bevirimat) is currently undergoing clinical trials with early favorable results.

POSTER 24

MOLECULAR MECHANISMS OF SIMIAN IMMUNODEFICIENCY VIRUS SIVagm RNA ENCAPSIDATION

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Primate lentiviruses are composed of several distinct lineages, including human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus SIVagm. The molecular mechanisms of viral RNA encapsidation have been studied in HIV-1 and HIV-2. However, very little is known about how SIVagm packages its RNA. Interestingly, HIV-1 and HIV-2 have significant differences in the mechanisms of viral RNA encapsidation. Therefore, the RNA packaging mechanisms of SIVagm cannot be predicted from the studies of HIV-1 and HIV-2. We sought to determine the molecular mechanisms of SIVagm RNA encapsidation by first examining the roles of the nucleocapsid (NC) zinc finger motifs on RNA packaging. Substitution mutations that abolished either of the zinc fingers severely reduced the packaging of SIVagm RNA, whereas replacing the CCHC motifs with other zinc finger motifs had varied effects, from sustaining the wild-type RNA packaging specificity to significant loss of such recognition. We then examined whether SIVagm has a preference to package RNA in *cis* by comparing the RNA packaging efficiencies of *gag* mutants in the presence of a wild-type vector. We found that RNAs from SIVagm *gag* mutants, including RNAs from Gag truncation mutants and packaging-defective Gag mutants, were efficiently packaged in the presence of wild-type RNA. Taken together, our data indicate that the SIVagm NC domain plays an important role in Gag-RNA recognition; furthermore SIVagm is distinct from the other currently known primate lentiviruses as destroying either zinc finger motif in the NC causes very drastic RNA packaging defects. Additionally, *trans*-packaging is a major mechanism for SIVagm RNA encapsidation.

[*] Equal contribution

POSTER 25

ROLE OF BASIC AND AROMATIC RESIDUES IN THE NUCLEIC ACID CHAPERONE ACTIVITY OF HIV-1 NUCLEOCAPSID PROTEIN

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Reverse transcription of the HIV-1 RNA genome involves multiple nucleic acid rearrangements. The minus-strand transfer step requires annealing of highly structured complementary regions called transactivation response region (TAR) RNA and TAR DNA. *In vitro* studies have demonstrated that annealing of these TAR elements is not spontaneous and is greatly enhanced by the HIV-1 nucleocapsid (NC) protein. NC is a chaperone protein that facilitates the rearrangement of nucleic acid structures to a thermodynamically more stable state, through a combination of nucleic acid aggregation and destabilization activity. Although the role of NC's zinc fingers in destabilization is well established, the role of other structural features is less well understood. In this work, we probed the role of basic and aromatic amino acid residues of NC in mediating TAR RNA/DNA hairpin (59-nucleotides each) annealing and nucleic acid aggregation. Simultaneous mutation of five basic residues (K3A/R7A/R10A/K11A/K14A) in the N-terminal domain of NC results in a protein with a severely reduced chaperone activity. Surprisingly, although the nucleic acid aggregating activity of this mutant is reduced relative to wild-type NC(1-55), it is greater than that of NC(11-55), an N-terminal truncation mutant lacking only 3 basic residues. Simultaneous mutation of basic residues in the linker region between the two zinc fingers (R29A/R32A/K33A/K34A) also has pronounced effects on the chaperone activity and on nucleic acid aggregating ability. Taken together, these results demonstrate that there is a direct correlation between the number of basic residues mutated and the effect on aggregation capability. In addition, the structural context of the basic residue mutations is an important factor for overall chaperone activity. The aromatic mutants, F16A, W37A and F16/W37A, also resulted in diminished activity, which may be due to reduced interactions with the exposed nucleobases. This hypothesis is supported by fluorescence anisotropy measurements, which demonstrate reduced binding affinity of these aromatic mutants to $(TG)_4$.

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POSTER 26

FUNCTIONAL COMPLEMENTATION OF TWO GAG MUTANTS DURING HIV-1 REPLICATION

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During HIV-1 assembly, Gag domains NC and p6 play important roles in RNA encapsidation and virus release, respectively. We have previously demonstrated that functional complementation occurs between an NC mutant that lost the specificity for viral RNA packaging, and a p6 mutant with reduced virus production. When expressed separately, these two mutants have abolished or severely reduced viral infectivity. However, when these two mutants were co-expressed at a 1:1 ratio, infectious viruses were produced efficiently.

Using this system, we sought to first determine the amounts of functional NC required to restore viral RNA packaging. Compared with wild-type viruses, our NC mutant packages 5% of the viral RNA. We transfected NC and p6 mutants at different ratios and analyzed the amounts of RNA packaged in the virions. Compared with wild-type virus, viral RNAs were packaged at 50% efficiency when the NC and p6 mutant DNAs were transfected at a 5:1 ratio, whereas viral RNAs were packaged at wild-type level when the NC and p6 mutant DNAs were transfected at a 1:1 or 1:5 ratio. Therefore, only a small portion (~20%) of the Gag needs to contain functional NC domain to generate a 10-fold rescue of viral RNA packaging (5% to 50% of wild-type level).

We then examined the virus titers generated from the NC and p6 mutants complemented at different ratios. We found that at 5:1, 1:1, and 1:5 ratios of NC and p6 mutants, virus titers were rescued at 4, 47, and 86% of the wild-type level, respectively. These results indicated that the replication of some mixed viruses was blocked at a post-RNA packaging step. We are currently characterizing the steps at which replications was blocked in these phenotypically mixed viruses.

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POSTER 27

DOMINANT EFFECT OF HIV-1 *GAG* POLYMORPHISMS AND TARGET CELL TYPE ON REPLICATIVE FITNESS AND DRUG RESISTANCE

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Regions of HIV-1 *gag* between p2 and p6/p6^{Pol} develop genetic diversity in HIV-1 infected individuals who fail to suppress virus replication by combination protease inhibitor [PI] therapy. To identify cell-specific and viral *gag-PR* determinants of replicative fitness and drug sensitivity, replication-competent recombinant viruses constructed with pre- and posttherapy *gag*-PR alleles were tested for replication and drug sensitivity in CXCR4- or CCR5-expressing peripheral blood mononuclear cells [PBMC]. A panel of sixteen recombinant viruses was generated and tested for replicative kinetics in the absence of PI and for susceptibility to indinavir [IDV] or ritonavir [RTV] by determining IC_{50}. P-glycoprotein [P-gp] activity was assessed by a Rhodamine 123 efflux assay in X4 and R5 PBMC. Site-directed mutagenesis combined pretherapy p2/p7^{NC} cleavage site, p7^{NC}, p6 and p6^{Pol} sequences with genotypic and phenotypic drug-resistant or -sensitive PR alleles. In the absence of PI, the posttherapy virus replicated to 4-9 fold lower levels than the pretherapy virus in X4 PBMC, but to similar levels in R5 PBMC. In the presence of PI, the posttherapy virus required 40-200 fold more drug to inhibit replication in R5 PBMC compared to X4 PBMC. P-gp activity was highest among $CD4^+$ lymphocytes that express CCR5 and CD45RO. Cell type and positions in *gag* exert a dominant effect on both replicative fitness and resistance to PIs. P-gp activity contributes to the higher PI levels required to inhibit replication in R5 PBMC as compared to X4 PMBC. Replicative capacity of viruses can be independent of susceptibility to PIs, while genotypic and phenotypic drug-resistant PR can exhibit susceptibility to PIs by changes in *gag*. Functional interactions between *gag* and PR predict that mutations in one region would cause conformational changes that modulate interactions between active site and substrate. These data support a model of structural organization of the Gag-Pol polyprotein prior to and during processing by PR. Understanding the functional linkage between Gag and PR could lead to developing novel therapeutic strategies to inhibit drug resistant viruses that are aimed at blocking structural conformations rather than the active site of PR.

POSTER 28

MODULATION OF HIV PRODUCTION BY THE GOLGI-LOCALIZED GAMMA EAR-CONTAINING ARF-BINDING (GGA) PROTEINS

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Human immunodeficiency virus type 1 (HIV-1) Gag is the major structural determinant of virus assembly either on the plasma membrane (HeLa and T cells) or in late endosomal compartments (macrophages). While the role of cellular factors in facilitating late stages of virus assembly and release is well established, very little is known about the targeting of Gag to the site(s) of virus assembly in T cells or macrophages. Here we report that the Golgi-localized gamma ear-containing Arf-binding proteins (GGAs) act as both positive and negative regulators of HIV assembly, apparently by affecting Gag trafficking to the plasma membrane. siRNA-mediated depletion of GGAs led to a moderate increase in HIV-1 release in a manner dependent upon the HIV-1 PTAP late domain. Since GGAs interact with Tsg101, the increase in virus release upon GGA depletion could be due to higher levels of Tsg101 available for virus budding. Over-expression of GGAs led to a reduction in HIV-1, equine infectious anemia virus (EIAV), and murine leukemia virus (MLV) particle production. Attempts to stage the block in virus production suggested that there is a significant decrease in membrane binding of Gag without any defects in Gag multimerization or total Gag synthesis. GGA over-expression also led to the accumulation of aberrant swollen endosome-like compartments in transfected cells, which in the case of GGA1 also trapped newly assembled particles. Electron microscopy data suggest that the defect induced by GGA over-expression is distinct from that induced by overexpression of full-length or truncated forms of Tsg101 or dominant-negative Vps4. These finding emphasize the importance of the GGA family of proteins as novel modulators of HIV-1 Gag trafficking. Further studies will be aimed at deciphering the precise role of the GGAs in Gag trafficking and virus particle production.

POSTER 29

UNIQUE FEATURES OF HIV-1 PROTEASE STRUCTURE AND DRUG-RESISTANCE AS REVEALED BY NMR

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Long-term solution NMR studies of the mature HIV-1 protease become feasible at high protein concentrations in the absence of inhibitor by using a pseudo wild-type that contains Q7K/L33I/L63I mutations to restrict autoproteolysis and C67A/C95A mutations to avoid protein aggregation mediated by cysteine-thiol oxidation[1]. Some unique aspects of the protease as revealed by NMR studies are as follows: (I) Protease dynamics by NMR have identified residues involved in (a) the flap motion and (b) conformational exchange in the terminal □-sheet region, both related to its function[2,3]. Dynamics parameters derived from NMR have been used to verify molecular dynamics simulation results. (II) Mutational and NMR structural studies reveal that folding and dimerization of the mature protease are independent events. The intra-subunit interaction between the conserved D29 and R87 residues is critical for establishing the very low equilibrium dissociation constant (K_d) of the mature protease; mutating these residues increases the k_d by > 4 orders of magnitude[4]. Darunavir, a recently approved clinical inhibitor which is designed to make a polar interaction with the conserved D29, is expected to be more effective against drug-resistant variants[5]. (III) Crystallographic analysis of the drug-resistant flap mutant F53L in the absence of inhibitor revealed a more-open flap conformation suggesting a novel mechanism of drug-resistance[6]. Even though F53L in the presence of inhibitor has so far failed to crystallize, recent NMR studies have shown that the F53L mutant forms ternary complexes with inhibitors DMP323 or darunavir at ~ 0.4 mM concentration, with a flap and terminal □-sheet conformation that are similar to those of the wild type protease-inhibitor complexes.

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POSTER 30

CRYSTAL STRUCTURES OF HIV-1 PROTEASE GUIDE INHIBITOR DESIGNS TO OVERCOME DRUG RESISTANCE

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The development of drug resistance is a serious problem for the worldwide epidemic of HIV/AIDS, despite the dramatic success of antiviral drugs such as the inhibitors of HIV-1 protease. Antiviral HIV-1 protease inhibitors, in clinical use since 1995, were developed with the aid of crystal structures of protease-inhibitor complexes. Although these drugs have greatly improved the life span of HIV-infected people, new inhibitors are needed to target the emerging resistant protease variants. Therefore, it is imperative to understand the molecular basis for drug resistance by analyzing the effects of amino acid substitutions on the structure and inhibition of HIV-1 protease. The first protease inhibitors were designed to maximize hydrophobic interactions with the wild type protease. Resistant variants of the HIV-1 protease appear on treatment with these drugs. Clinical isolates with high level resistance to protease inhibitors commonly show mutations in one or more of the active site residues D30, V32, G48, I50, V82, and I84, and/or non-active site residues such as L90. Consequently, a second generation of antiviral protease inhibitors has been developed with the aid of crystallographic and kinetic analysis of protease mutants containing these substitutions. The design goal, based on similarities to the protease interactions with substrates, is to incorporate additional interactions with main chain atoms and conserved regions of the protease that cannot easily be eliminated by mutation. This strategy was applied in the design of the potent new antiviral inhibitors, darunavir, GRL-06579A and GRL-98065. These inhibitors have been analyzed in their complexes with the protease and the common resistant variants. The observed changes in protease structure and activity are discussed in relation to the potential for development of resistant mutants. Our high resolution structural and inhibition data for the recently approved antiviral drug darunavir have helped to understand its effectiveness on drug resistant HIV and demonstrate the success of this approach.

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POSTER 31

STRUCTURAL ANALYSIS OF NEWLY DESIGNED HIV-1 PROTEASE INHIBITORS

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The development of the HIV-1 protease inhibitors is regarded as a major success of structure-based drug design. Despite this remarkable success, there is still much concern regarding the treatment of AIDS, largely because of the emergence of the HIV mutants that resist current therapy. All the current HIV-1 protease inhibitors were designed to inhibit primarily a single variant of HIV-1 protease. Structural analysis of various complexes of protease with drugs and the substrates show that most primary active site mutations do not extensively contact substrates, but are critical to inhibitor binding. It is proposed that the inhibitors that lie within substrate envelope are less prone to develop resistance. To test this model, in collaboration with different academic groups, inhibitors were designed and synthesized to fit within substrate envelope. The binding affinities were measured for all the inhibitors and the inhibitors with the best binding affinities were crystallized with the wild-type protease. The salient features of the intermolecular interactions of these newly designed inhibitors with the protease will be presented.

POSTER 32

STRUCTURAL INSIGHTS INTO THE VARIED SPECIFICITY OF I50L IN HIV-1 PROTEASE

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The emergence of drug resistance in HIV-1 protease in response to antiviral therapy is a compounding problem in the fight against AIDS. Although the newly introduced protease inhibitors are highly potent, inhibition is still thwarted by the development of new mutations specific to these drugs. I50L is one such mutation that occurs in patients treated with atazanavir (ATV). Interestingly, this mutation is known to cause hypersusceptibility to other protease inhibitors such as amprenavir and darunavir. In this study we are utilizing crystallographic and thermodynamic data to elucidate this varied specificity.

POSTER 33

STRUCTURAL ANALYSIS OF HIV-1 CRF01_AE PROTEASE IN COMPLEX WITH THE SUBSTRATE P1-P6

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The homo-dimeric aspartyl protease of human immunodeficiency virus-1 (HIV-1) is a key target in anti-HIV drug development as it is essential for the processing of Gag and Gag-Pol polyproteins in the viral maturation process. However, the recognition of other HIV-1 clades and circulating recombinant forms (CRFs) have given rise to questions on the efficacy of currently available therapeutics which were designed originally against clade B protease. No structural information is available on non-B clade proteases and as a result very little structural knowledge is known on how substrate and inhibitor binding takes place. We report here, a crystal structure of CRF01_AE in complex with the p1-p6 substrate determined to a resolution of 2.5Å. Preliminary models built from this data set indicate a significant change in the flap hinge region between the CRF01_AE protease when compared to the subtype B protease in complex with the p1-p6 substrate. Further refinement of this data set may provide new insights into differences in how substrates interact between clade B and CRF01_AE.

POSTER 34

COMPARISON OF RESISTANCE MECHANISMS OF HIV-1 SUBTYPE C PROTEASE: NELFINAVIR AND ATAZANAVIR RESISTANT VARIANTS

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Nelfinavir (NFV), a first generation protease inhibitor (PI), is extensively used on the African continent where subtype C virus is the dominant subtype of the HIV/AIDS epidemic. Atazanavir (ATV) is a new, very effective PI and its potential recommends it for wide use. Only limited information about subtype C protease (PR-C) and PI resistance is available to understand the mechanisms through which the naturally occurring polymorphisms in HIV-1 PR-C might render this enzyme less susceptible to the inhibitory actions of PIs. Kinetic analysis of PR-C and NFV- and ATV-resistant variants was performed to determine the K_i values for all the mutants against eight clinically used PIs. PR-C showed similar K_i values and a slight decrease in catalytic efficiency, when compared to that of subtype B (LAI) protease (PR-B) for all PIs. Addition of NFV resistance mutations within PR-C had variable effects. The double mutant N88D/L90M of PR-C showed an increase of K_i values for NFV, saquinavir (SQV) and indinavir (IDV) of 8-, 28-, and 50-fold, respectively when compared to PR-B. On the other hand, the D30N/L90M combination was more advantageous for PR-B with a slightly lower binding affinity for IDV and SQV when compared with the same variants of PR-C. Two double mutants (D30N/N88D, D30N/L90M) of PR-C preserved 60% of catalytic efficiency of native PR-C, while the same mutants of PR-B exhibited a significant decrease in their catalytic activity – about 70%. The I50L mutant of PR-C exhibited a 2-3-fold increase in K_i values for ATV and lopinavir (LPV) when compared with PR-B harboring the same mutation, with no significant changes in the catalytic efficiency values.

Conclusions: 1) PR-C and PR-B have similar susceptibility for the inhibitors in clinical use. 2) The naturally occurring polymorphisms in the PR-C could provide for a greater level of resistance upon the acquiring both the L90M and N88D mutations. 3) The main way the virus overcomes the inhibitory effect of NFV appears to be through preserving the catalytic efficiency of the enzyme while the primary route to overcome the unfavorable effects of ATV is by decreasing the binding affinity of the enzyme to the inhibitor.

POSTER 35

THE ROLE OF HTLV-1 GAG UBIQUITINATION IN ITS INTERACTIONS WITH COMPONENTS OF THE MULTIVESICULAR BODY BIOGENESIS PATHWAY DURING VIRUS BUDDING

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Multivesicular bodies (MVB) are part of the pathway to lysosomal degradation for transmembrane proteins tagged by mono- or di-ubiquitination. Retroviruses appropriate the cell's machinery for vesicle formation to assemble and bud. A peptide motif in Gag called the late assembly domain (LD) interacts with various MVB protein complexes; the interaction depends on the peptide sequence of the virus LD. The role of ubiquitination, which is frequently observed on late domain containing viral proteins, is unclear and may differ among viruses and late domains. The HTLV-1 LD consists of two motifs located at the C-terminus of the MA domain and has the sequence PPPYVEPTAP. Mutation of the PPPY sequence significantly reduced virus budding and infectivity, and abolished ubiquitination of MA in virus particles and cellular extracts. Mutational analysis showed that the lysine residue at position 74 is the only substrate for ubiquitination in MA and that the K74R mutation reduced virus budding and infectivity by about 50 %. The PPPY motif interacted with the WW domains of the E3 ubiqitin ligase, WWP1. Expression of WWP1 with a truncation of the HECT domain showed a strong dominant negative effect on the HTLV-1 budding, which was not seen with an enzymatically inactive, full-length WWP1 molecule. This finding suggests that the HECT domain of WWP1 plays an important part in HTLV-1 budding by mechanisms in addition to its ubiquitination activity. Mutations in the PTAP motif also reduced budding by only 50 %. DN versions of TSG101, the ESCRTI component that connects proteins with PTAP motifs to the MVB pathway, inhibited HTLV-1 budding in Hela cells but not 293T cells. Overexpression of wildtype and mutant HRS, the cellular connector of mono-ubiquitinated proteins to TSG101, reduced HTLV-1 budding, while siRNA mediated downregulation of HRS enhanced virus release. Whether this effect resulted from a gross disturbance of the MVB machinery or is specific for HTLV-1 release is under investigation. Expression of wildtype or mutant AIP had no effect on HTLV-1 budding, while DN-VPS4 resulted in complete abrogation of virus release.

POSTER 36

CHARACTERIZATION OF HIV ENVELOPE CLONES FROM PATIENTS WITH REDUCED SUSCEPTIBILITY TO VICRIVIROC REVEALS PATIENT SPECIFIC MUTATIONAL PATTERNS IN GP120

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Background: Development of resistance to the CCR5 antagonist vicriviroc (VCV), an allosteric inhibitor of the gp120 and CCR5 interaction, is poorly understood. To better define the genotypic correlates of VCV susceptibility, detailed analysis was performed on envelope genes from 4 patients enrolled in a VCV clinical study who experienced viral breakthrough.

Methods: gp160 genes amplified from baseline and end of treatment (EOT) plasma samples were cloned, sequenced and tested for susceptibility to VCV; measured as percent of maximal viral suppression (PMS) achieved relative to untreated controls (PhenoSense assay).

Results: Viral pools generated from baseline samples from each patient were susceptible to VCV, with PMS values of > 94%. However, clonal analysis revealed that some pools consisted of a mixture of viruses with a range of PMS values (44% to 100%). Pools from EOT samples exhibited reductions in PMS relative to baseline (range 35 – 85%) and individual EOT clones also exhibited a wide range of susceptibilities to VCV (12%-100% PMS). Genotypic analysis of envelopes from individual patients showed that multiple amino acid changes occurred throughout gp120 and were more frequent in the V3-loop as well as in V1/V2 and V4 regions. Although specific mutations in the V3 crown and adjacent distal sequences correlated with resistance to VCV in some clones, V3 mutations alone were not predictive of phenotype, suggesting that mutations in other regions of gp120 also contributed to variation in susceptibility. The specific mutational patterns observed were unique for each patient and could not be used to predict the susceptibility of clones from the other patients studied. Phylogenetic analysis showed that in 3 of 4 patients, gp120 baseline sequences were more heterogeneous than sequences from EOT samples, suggesting VCV exerted selective pressure on viral evolution.

Summary: Resistance to VCV in these 4 patients was associated with multiple sequence changes in gp120, with changes occurring most frequently in the V1/V2, V3 and V4 regions. Mutational patterns were patient specific and could not predict resistance from gp120 sequences from the other patients. Additional phenotypic and genotypic data will be needed to assess the possibility of generating genotypic resistance models for VCV and other CCR5 antagonists.

POSTER 37

GENOTYPIC ANALYSIS OF ENVELOPE SEQUENCES OF PATIENTS ENROLLED IN THE CCR5 INHIBITOR VICRIVIROC CLINICAL TRIAL

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Introduction: CCR5 inhibitors are a new class of antiretrovirals targeting HIV entry. Little is known about the mechanisms leading to resistance to this new class of drugs. Understanding the variation in the envelope sequences of HIV-infected patients receiving these inhibitors is the first step to better characterize viral evolution under the selective pressure of these drugs. We present envelope sequence analysis for 25 patients enrolled in a Phase II Vicriviroc clinical trial, 21 of whom with clinical failure.

Methods: Envelope genes from plasma samples from baseline and end of treatment (EOT) were amplified and sequenced. Gp 160 sequences were aligned and analyzed using Sequencer 4.6, and Bioedit software. Phenotypic analysis was performed using the PhenoSense Assay. Nine subjects were enrolled in the the 25 mg group, 9 in the 50 mg group, 5 in the 75 mg group, and 2 in the placebo group.

Results: The genetic variation between baseline and treatment varied considerably from subject to subject and did not correlate with either phenotypic susceptibility, viral tropism, or treatment dose. Variation occurred throughout gp120, particularly at glycosylation sites within C1/V1, V2 and V4. Mutations were seen in gp41 in all subjects but one, mainly near the leucine zipper domain. The 4 patients with the lower number of mutations (less than 5 mutations over the entire gp160) were on the 25 mg regimen and did not harbour any mutations in the V3 loop. Of the 3 subjects who had a switch in tropism from R5 to X4, one who was on placebo did not show any evolution within the V3 loop, the other two had mutations adjacent to the crown of the V3 loop and one of them harboured the most variation in his entire gp160 gene.

Discussion: During CCR5 inhibitor therapy, viral evolution occurs throughout the entire gp160. Sequence variation does not predict phenotypic susceptibility. Some subjects failed therapy with very little evidence of env evolution and selective pressure on the genome does not seem to be related to treatment doses. Additional detailed analysis will be performed to evaluate the predictive value of genotypic analysis.

POSTER 38

MUTATIONS AND POLYMORPHISMS IN THE GP41 OF THE HIV-1 FROM T20 NAÏVE PATIENTS RECEIVING HAART

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In many patients with HIV infection, therapy does not result in complete suppression of HIV replication and leads to the failure. Like this, the failure on treatment requires the development of new therapeutics alternatives. T-20 is a synthetic 36-aminoacid that binds to the HR1 domain and inhibits entry of HIV-1 into host cells. The gp41 HR1 region sequencing has been identified a contiguous three-aminoacid residue sequence within HR1 (positions 36-38) that is critical for inhibition of efficient association between HR1 region and T-20 peptides. Genotypic and phenotypic analysis from HIV-1 strains and observation of these resistance-associated mutations are important to direct choose of antiretroviral scheme during the treatment of patients that present fusion inhibitors resistant virus. 62 HIV-infected patients with a CD4+T cell count<350 cell/mm^3 who were receiving at least a 3-drug HAART regimen with HIV RNA levels>10.000 copies/mL plasma and were eligible for enrollment. The gp41 gene was amplified by PCR nested and sequenced using primers at positions 7789-7816 and 8265-8294 relative to HXB2 sequence. Neighboor-joining Trees were generated and all sequences were submitted to Blast at Los Alamos HIV Sequence Database. Of the 62 samples 57 (92%) were of subtype B, four (6,5%) subtype F and one (1,6%) subtype C on the gp41. Analysis of the sixteen aminoacids involved in resistance revealed that all had identical residues at critical 36(G), 37(I) and 38(V) positions. Other changes were detected in several positions: 3,23% in 30(A); 25,8% in 32(Q); 16,13% in 42(N); 1,61% in 43(N); 3,23% in 44(L); 14,52% in 46(R). The present study demonstrates the high conservation of the amino acids 36-38 of gp41 region. Our data confirms the previews findings that primary T20 genotypic resistance is not frequent, especially in the GIV motif, a critical region for resistance These findings provides the importance of the study of the gp41 region of the HIV-1 in patients failure in treatment with the HAART. T20 may be used in association with HAART and to represent a new treatment strategy.

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POSTER 39

CHARACTERIZATION OF THE ROLE OF TWO ADDITIONAL HIV-1 REVERSE TRANSCRIPTASE MUTATIONS IN THE REGULATION OF NNRTI RESISTANCE

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We recently demonstrated the association of two additional mutations (I135T and H221Y) in HIV-1 reverse transcriptase with the NNRTI-treatment and NNRTI-resistance mutations. Thus, we characterized the role of such mutations in the regulation of NNRTI-resistance. 1904 HIV-1 *pol* sequences from 758 drug-naïve patients, 592 NRTI-treated/NNRTI-naïve patients and 554 NRTI+NNRTI-treated patients were analyzed. The association of mutations with NNRTI-treatment was assessed by Fisher's exact test. Covariation analysis was based on the binomial correlation coefficient and hierarchical clustering. The phenotypic impact of mutations on NNRTI-resistance was determined using sequences from Stanford HIV Drug-Resistance Database and Arevir Database, by Wilcoxon's test and support vector regression (SVR). Molecular dynamics simulations (MDS) were performed by using the Gromacs software.
I135T, polymorphism in drug-naïve patients (32% prevalence), occurred in 39% and in 50.2% of NVP-treated and EFV-treated patients, respectively, was positively correlated with K103N and occurred in SVR models within the top-20 determinants for NVP-resistance. When together with K103N, I135T was also associated with 3.4-fold increase in K103N/EFV-resistance. MDS showed that in presence of I135T, residues 103 and 188 are closer, and there is a higher frequency of hydrogen-bond occurrence between 103N and 188Y, suggesting that 135T may stabilize the closure of the NNRTI binding pocket induced by K103N. In addition, the presence of I135T at baseline (before NNRTI-treatment) correlated with K103N at NNRTI-failure ($P=0.028$).

Differently, H221Y, rare in drug-naïve patients, occurred in 10.3% and in 6% of NVP-treated and EFV-treated patients, respectively, was positively correlated with Y181C, and occurred within the top-20 determinants for NVP- and EFV-resistance. In particular, the co-presence of H221Y+Y181C was associated with 12.4 fold increase in Y181C/NVP-resistance.
In addition, H221Y showed antagonistic correlations with the TAMs2 ($p<0.01$), and, interestingly, its co-presence with the TAMs2, alone or together with Y181C, was associated with higher ZDV susceptibility.

In conclusion, our study reinforces the complexity of NNRTI resistance and the non-negligible interplay between NRTI- and NNRTI-selected mutations. Mutations beyond those currently known to confer resistance should be considered for improved prediction of clinical response to antivirals and for the maintenance of potential efficacy of new-generation NNRTI.

POSTER 40

VIROLOGICAL RESPONSE TO HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) AND THE EMERGENCE OF DRUG-RESISTANT MUTATIONS IN HIV-2 AND HIV-1/HIV-2 DUALLY INFECTED PATIENTS IN THE GAMBIA

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Background: Limited data exists on antiretroviral therapy (ART) and drug resistance in HIV-2 and dually-infected patients. It has been reported that various HAART regimens fail to suppress HIV-2 viral replication to undetectable levels. With increasing access to ART in Africa due to the Global Fund, it is important to understand the response of these patients to therapy; document clinically relevant resistance mutations and develop cheaper drug-resistance assays that are more sustainable for use in resource-poor settings.

Materials and Method: Twenty patients, 12 HIV-2 and 8 dually-infected patients on triple therapy (AZT+3TC+Lop/r) were monitored to study their immunological and virological response to therapy. Patients with viral rebound were genotyped by sequencing. We developed and evaluated an HIV-2 Oligonucleotide Ligation Assay (OLA) to detect known drug-resistance mutations.

Results: Viral load was reduced to undetectable levels in all, but two HIV-2 patients. During follow up, 3 patients, with initial undetectable viral load on HAART, experienced viral rebound. Sequence data from these 5 patients showed the presence of 7 documented HIV-1 drug-resistance mutations present naturally in these HIV-2 viruses. One known HIV-2 mutation, M184V, against Lamivudine, was observed in one patient. New mutations that seem to emerge as a result of drug pressure were also observed.

Conclusions: HIV-2 response to HAART is similar to HIV-1 response; with viral loads reduced to undetectable levels. We observed documented and as well as potential new drug-resistance mutations, however phenotypic assays are needed to determine their clinical relevance. We successfully developed and evaluated a simple, economical and sustainable assay to detect the M184V mutation in HIV-2.

POSTER 41

ANALYSIS OF CO-EVOLUTION BETWEEN MUTATIONS IN PROTEASE INHIBITOR RESISTANCE AND IN GAG

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Background and Objective: Protease inhibitor (PI)-resistant mutations reduce HIV-1 processing of precursors to $Pr55^{Gag}$ and $p160^{Gag\text{-}Pol}$. As a result, viruses with PI-resistant mutations often demonstrate lower replication capacity than that of wild-type viruses. However, the acquisition of PI-resistant mutations exerts strong selective pressure on the virus to recover its replication capacity, and many mutations are introduced into Gag, the substrate of viral protease. This phenomenon is known as co-evolution of the protease and Gag. To better understand the mechanism of co-evolution in PI-resistant viruses, we used Single Genome Sequencing to analyze serially collected samples from HIV-1-infected patients.

Methods: Plasma samples were collected serially from HIV-1-infected patients (subtype B) who had received anti-HIV treatments between April 1998 and August 2002. Reverse nucleotide sequences of Gag-protease (Pr)-reverse transcriptase (RT) fragments (3Kbp) were analyzed by single genome sequencing (Palmer et al., 2005). The resulting sequences were analyzed by the CoMap program (Dutheil et al., 2005), which detects significantly interfering loci within molecules. Sequences were analyzed for 6 different combinations: 3 intramolecular (Gag, Pr and RT) combinations and 3 intermolecular (Gag-Pr. Gag-RT and Pr-RT) combinations. The analyses were performed with two parameter settings. One setting was a hypothesis of interference, and the other was the null hypothesis of independence. The results of both analyses were compared to eliminate false-positive outcomes of co-evolving loci.

Results & Conclusion: Plasma samples were collected at 7 times (at 1- to 12-month intervals), and 82 Gag-protease-RT clonal sequences were analyzed. CoMap analyses showed that the Pr mutation E35D was significantly linked with the Gag p1-p6 cleavage site mutation P453L. In addition, Pr-Pr intramolecular analyses revealed that Pr mutation E35D was linked with the nelfinavir-resistant mutations D30N and N88D. Interestingly, we also found 7 co-evolving sites between Gag and RT. These results provide evidence that Gag, protease, and reverse transcriptase molecules are interfering in their evolution and selective process under antiretroviral treatment.

POSTER 42

ROLE OF RECOMBINATION-DRIVEN HUMAN SEQUENCE TRANSDUCTION ON THE GENESIS OF MULTIPLE-DRUG RESISTANT MUTANT IDENTIFIED IN JAPAN

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While most of genetic variation of HIV-1 arises by stepwise accumulation of point mutations, genetic recombination also contributes to genetic diversity and occurs about 10-fold more frequently than base substitution. It is known that retroviral recombination results from template switching during reverse transcription and generally occurs in regions of high sequence similarity between the two co-packaged genomes. However, non-homologous recombination, often guided by microhomology between donor and acceptor templates, can also occur and generates various types of deletions and duplications as well as so-called "insertion-in-a-deletion" and "insertion-in-a duplication" mutations as a result of a series of non-homologous crossovers. It is also known that host sequences can template insertions, as postulated by models for oncogene transduction.

In the present study, we examined an unusually long insertion mutation in an HIV-1 isolate from a Japanese child (NH3) identified in a unique intrafamilial CRF01_AE infection case, for elucidating its likely origin. The child (NH3) acquired HIV-1 CRF01_AE strain through maternal transmission from his mother (NH2), who was infected from her husband NH1. NH1 was infected heterosexually in Thailand in late 1990s. During highly active antiretroviral treatment in NH3, multidrug resistant virus (designated 99JP-NH3-II) with unusual 33-nucleotide insertion in the β3-β4 loop in reverse transcriptase was isolated (Sato et al. *J. Virol.* 75 (12): 5604-5613, 2001).

Support for possible non-viral origins of the 33-base insertion came from its striking nucleotide composition. Whereas typical HIV sequences has a <40% G+C content, this insert was 67% G+C content, implying the possible human origins for this insert. This insert sequence was BLASTed to query in databases. We found that the closest GenBank match to this insert was a 30/31-base match to repetitive sequence on human chromosomes 17 with an expect score of 0.00005. That the virus-human sequence match included a few bases downstream of the insertion was consistent with the possibility that microhomology guided recombination between HIV-1 and the BLAST-identified human sequence generated this insertion.

Based on these observations and the nature of the mutations, we postulated the plausible mechanism of this insertion mutation as follows. Briefly, an HIV-1 provirus was established on chromosome 17 just upstream of the putative insert-coding sequences. Viral polyadenylation signal read-through generated a chimeric HIV-human RNA that became encapsidated. During subsequent reverse transcription, microhomology-guided template switching between portions of the RT gene and human sequences on the read-through RNA generated the observed insertion-in-a-duplication structure. The plausible mechanism of multidrug resistance in this mutant strain is also discussed. (We acknowledge Drs. Hironori Sato and Teiichiro Shiino for their prior study)

POSTER 43

DRUG RESISTANCE MUTATIONS IN THE POLYMERASE CATALYTIC DOMAIN NEGATIVELY AFFECT THE RNASE H ACTIVITY OF HIV-1 REVERSE TRANSCRIPTASE

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A unique multi-drug resistant human immunodeficiency virus type 1 (HIV-1) subtype E (CRF01_AE) with an exceptionally long 11 amino acid insertion in the □4-□5 loop of reverse transcriptase (RT) was previously isolated (Sato H, et al., *J Virol* 2001). The insertion confers a 5.2-fold greater resistance to 3TC when present alone. However, in the context of the M41L, L210W, and T215Y mutations, which renders the virus 36-fold more resistant to AZT, the insertion augments both AZT and 3TC resistance and confers d4T, ddI, and ddC resistance. The mechanism of augmentation of MDR has been unclear. By a biochemical approach, we demonstrated that the combination of the point mutations with the insertion cooperatively reduced RNA-dependent DNA polymerase (by 5.6-fold), DNA-dependent DNA polymerase (3.3-fold), and RNase H activities (1.6-fold), whereas template switching activity was significantly increased as compared to wild type RT. This is the first demonstration that mutations conferring resistance to nucleoside RT inhibitors at or near the polymerase catalytic site of RT reduce RNase H activity. These phenotypes might be partly due to the decreased affinity of the MDR RT to substrates (62.5% compared to the WT enzyme). These data suggest that nucleotide excision plays a role in the augmentation of drug resistance since the reduction in polymerase activity should increase the chances of removing incorporated chain terminators. Biochemical and virological analyses indicate that the parallel reduction of the RT polymerase and RNase H activities has a selective advantage *in vivo*.

POSTER 44

INDEPENDENT PATTERNS OF SEQUENCE VARIATION IN HIV-1 *PRO* AND RT ARE FACILITATED BY FREQUENT RECOMBINATION

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Backgound: HIV-1 replication is both rapid and error-prone. Recombination events occurring during reverse transcription permit reassortment of new mutations. Prior to initiating antiretroviral therapy, genetic variation in *pro* and *pol* are subject to immune and purifying selective forces. To investigate whether *pro* and RT vary independently or in a linked fashion in response to selection, we compared sequence variation and phylogenetic relationships of *pro* and RT in chronically infected drug-naïve individuals.

Methods: Individual HIV-1 *pro-pol* sequences were obtained by single genome sequencing using endpoint dilution/PCR amplification; 15-20 amplicons (PR and nt 1-1200 of RT) per plasma sample were aligned using Clustal W and subjected to phylogenetic analyses. A total of 950 single genome sequences from 14 HIV-1 infected, drug naïve individuals were were generated from samples obtained over 1-14 years.

Results: Analysis of HIV-1 sequence variation revealed nucleotide diversity of *pro* and RT was comparable within individuals. Longitudinal analyses using samples obtained over 1-14 years revealed the presence of relatively homogenous HIV-1 *pro* and RT populations; bootstrap analysis did, however, detect several distinct *pro* and RT phylogenetic relationships (boostrap values > 85%) and additional analyses revealed population shifts in *pro* occurred prior to RT within several individuals, suggesting independent sequence variation occurred. To investigate whether recombination potentially contributed to independent *pro* and RT variation, we investigated the distribution of recombination intervals across *pro-pol.* Recombination was frequent with a mean of 11.6 recombination events per amplicon. Mapping the midpoints of recombination intervals revealed that crossovers were not uniformly distributed throughout *pro-pol*. Specific hot spots for recombination were not identified, but three-fold differences in the distribution of crossovers were noted throughout *pro-pol*, indicating non-uniform recombination or selection of recombinants. The mean size of recombination intervals (75.3 nt) was sufficiently small to permit frequent recombination between *pro* and RT.

Conclusions: In chronically infected individuals, gene-specific sequence diversity in *pro* and RT is comparable and stable over time. Phylogenetic analyses suggest that *pro* and RT may vary independently, even in the absence of drug selection pressure.

POSTER 45

EFFICIENT BIOCHEMICAL AND CELL-BASED REVERSE TRANSCRIPTASE ASSAYS FOR CHARACTERIZATION OF NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

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Reverse transcriptase (RT) plays an essential role in the HIV replication process, which converts a single strand RNA into a double strand DNA via polymerase and RNase H activities. Inhibition of reverse transcriptase has been one of the primary therapeutic strategies in suppressing the replication of HIV-1. This study presents a homogeneous and sensitive non-radioactive assay for the characterization of non-nucleoside reverse transcriptase inhibitors (NNRTIs). This electrochemiluminescence (ECL) assay employs a ruthenylated dUTP as the dNTP which can be incorporated into the DNA strand through RT polymerase activity. The concentration of the enzymes (WT, K103N and Y181C) required for the assay can be as low as 1 pM, enabling us to evaluate inhibitors with low picomolar potency. Furthermore, the assay is capable of detecting endogenous RT activity in viruses released into cell medium, and thus can be used to evaluate the NNRTI potency against the RT present in lysed viruses. As a result, the assay can be applied to monitor the resistant mutations developed by viruses during NNRTI treatment. To illustrate the use of these assays, characterizations of several NNRTIs are also described.

POSTER 46

MECHANISMS OF FIV RELEASE FROM INFECTED CELLS

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Infection of domestic cats with feline immunodeficiency virus (FIV) is an important and relevant non-primate model for the pathogenesis of HIV in humans. For example, FIV replication has been shown to be sensitive to primate Trim5□, HIV-based protease inhibitors, and possibly APOBEC3G. Vaccination of cats with inactivated FIV virions or HIV-1 p24 is also highly protective against FIV infection. Compared to HIV however, the cellular and molecular mechanisms that govern many aspects of the FIV infectious cycle, including infectivity, integration, Gag targeting, and virus assembly/release and maturation are poorly understood. Our aim is to further validate FIV as a relevant model for both antiretroviral and gene therapy using a comparative, cell biological approach. It has been well established that retroviruses use short peptide motifs called “late domains” to usurp cellular endosomal sorting (ESCRT) machinery and promote virus release. For example, a PTAP motif in HIV-1 Gag interacts directly with ESCRT I via Tsg101. Perturbation of ESCRT or Tsg101 function with dominant-negative mutants severely restricts HIV-1 release. We propose that FIV utilizes a similar motif (PSAP) in the same manner. Expression of a PTAP-binding fragment of Tsg101 (TSG-5’) inhibited FIV release in HeLa cells. Stable TSG-5’ expression in feline CrFK cells inhibited FIV replication. FIV release was also sensitive to expression of other dominant-negative forms of ESCRT-related components. Mutagenesis of the PSAP motif inhibited FIV release and replication. In all cases, defects in virus budding were confirmed by electron microscopy. Another ESCRT-related factor, Alix, is used by the non-primate lentivirus EIAV for virus release and may also promote HIV release in some cell types. Mutagenesis of a potential Alix binding site motif (LxxL) in FIV Gag did not affect FIV release in our studies, but may affect virus replication in feline T-cells. Our data suggest that FIV relies predominantly on the PSAP motif in Gag as a likely Tsg101 binding site to exploit the cellular endosomal sorting machinery as a platform for virus release in HeLa cells, and this mechanism of virus release is highly conserved in feline cells.

POSTER 47

HUMAN T CELL LEUKEMIA VIRUS TYPE 1 GAG TARGETS INNER LOOP OF THE TETRASPANINS CD82 AND CD81

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The tetraspanin superfamily (TM4SF) protein, CD82, plays important roles in organizing membrane protein complexes, modulating integrin function, and is involved in T cell adhesion. CD82 contains a large and a small extracellular loop with its N-terminus, C-terminus and short inner loop exposed to the cytoplasm. We have shown that the matrix domain of HTLV-1 Gag targets tetraspanin containing membrane microdomains at the cytoplasmic face of the plasma membrane and Gag can be reciprocally co-immunoprecipitated with CD82; we hypothesized that this association would bring virion components to the adhesion interface and facilitate HTLV-1 assembly, release, and transmission at the site of cell-cell contact. To map the interaction motifs in CD82, we generated site-directed mutations in the various domains of CD82 and used co-immunoprecipitation and co-localization approaches to examine interactions with Gag. Among the intracellular domains of CD82, only mutations of conserved amino acids in the inner loop diminished its association with Gag. In addition, mutations of juxtamembrane cysteine residues that are sites of palmitoylation abolished Gag-CD82 interaction. Thus, the inner loop of CD82, flanked by palmitoylated cysteines, is essential for CD82 interaction with HTLV-1 Gag. In addition, HTLV-1 Gag interacted with the inner loop of CD81, suggesting that Gag can target other tetraspanins via conserved inner loop domain. Unlike HTLV-1 Gag, HIV-1 Gag did not associate with CD82 at the plasma membrane.

POSTER 48

CROSS-CLADE INHIBITION OF HIV-1, SIVCPZ AND HIV-2 REVERSE TRANSCRIPTASES BY NUCLEIC ACID APTAMERS

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RNA and ssDNA aptamers have been selected that specifically and potently inhibit the reverse transcriptase (RT) from HIV-1subtype B (Kd values in the low nM to high pM range). To evaluate RT inhibition across multiple clades, we assembled a panel of recombinant RT's from phylogenetically diverse lentiviral isolates, including representatives from HIV-1 group M, HIV-1 group O, SIVcpz and HIV-2. After establishing baseline DNA- and RNA-dependent DNA polymerization and RNase H activities for all ten RT's — as well as inhibition by two NRTI's and two NNRTI's — dose-response curves for each enzyme were established for six nucleic acid aptamers.

For the **two family 1 pseudoknot RNA aptamers**, natural resistance was essentially all-or-none. In contrast, natural resistance to the **two family 2 pseudoknot RNA aptamer** was much more heterogeneous, both in degree (gradation of IC_{50} values) and distribution across clades. A single amino acid change was sufficient to confer resistance to the family 1 pseudoknot aptamers, while this same alteration did not correlate with resistance to family 2 pseudoknots aptamers. The **ssDNA aptamer RT1t49** inhibited all of the recombinant RT, with half-maximal inhibition occurring in the low- to mid-nM range for both polymerase and RNase H activities. This is the first demonstration of universal inhibition of lentiviral RTs by a nucleic acid aptamer and supports previous reports suggesting that resistance to RT1t49 may be exceptionally infrequent. In contrast, **ssDNA aptamer RT8** inhibited only the RT from subtype B. These results demonstrate that structural diversity among aptamer inhibitors translates into a non-overlapping spectrum of mutations that confer resistance, likely due to differences in atomic-level contacts with RT. Finally, both the RNA and ssDNA aptamers demonstrated synergy with NRTI compounds and were additive when used in combination with NNRTIs, suggesting that they are compatible as adjuvants to chemotherapy of HIV infection.

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